Mechanisms Responsible for Differential Bactericidal Activities of Human and Rabbit Complement Against *Neisseria Meningitidis*

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ABSTRACT

Polysaccharide conjugate vaccines are available to prevent disease caused by *Neisseria meningitidis* serogroups A, C, W and Y. Meningococcal vaccine efficacy is assessed in clinical trials using serum bactericidal assays (SBAs). Baby rabbit serum is usually used as the source of complement for SBAs as it lacks endogenous bactericidal activity compared to human serum. Previous studies have shown that SBA activities determined with rabbit (rSBA) and human (hSBA) complement correlate poorly, possibly due to different interactions between antibody subclasses and the complement source, and species-specific interaction of *Neisseria meningitidis* with complement regulators. The aim of this project was to investigate the mechanisms responsible for differential bactericidal activities of human and rabbit complement against *Neisseria meningitidis*.

The serum concentration of polysaccharide-specific antibody subclasses was measured following vaccination with quadrivalent meningococcal polysaccharide vaccines; data showed that the concentration of polysaccharide-specific IgG1 antibody correlated most significantly with hSBA titres whereas the concentration of polysaccharide-specific IgM antibody correlated most significantly with rSBA titres. The interaction of human IgM and IgG subclasses with human and rabbit complement was compared at the level of C1q and C3 using both binding and functional assays. These data define important differences in the ability of human antibody subclasses to fix human and rabbit complement. Specifically, polysaccharide-specific IgM contributes significantly more to bactericidal titres in rSBAs compared to hSBAs. As a consequence, rSBAs produce misleadingly high titres in individuals with large IgM responses to vaccination. Using a series of pathway-specific inhibitors, it was shown that the alternative pathway contributes significantly more to the bactericidal activity of rabbit complement towards *Neisseria meningitidis* than human complement.

This project provides significant insight into the difficulties and challenges associated with the interpretation of rSBA data, enhances the understanding of antibody responses to meningococcal vaccines and will support improvements in the development and testing of meningococcal vaccines.

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ABBREVIATIONS

- ADCC Antibody-Dependent Cell-Mediated Cytotoxicity
- AID Activation Induced Cytidine Deaminase Enzyme
- AP Alternative Pathway
- BBB Blood-Brain Barrier
- BCECF-AM 2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester
- BCR B Cell Receptor
- BRS Baby Rabbit Serum
- BSA Bovine Serum Albumin.
- C1INH C1 Inhibitor
- C4BP C4 Binding Protein
- CD Cluster of Differentiation
- CDC Complement-Dependent Cytotoxicity
- CFD Complement Fixation Diluent.
- CFR Case Fatality Rates
- CFU Colony Forming Units
- CH Constant Heavy Chain Domain of immunoglobulin
- CI Confidence Interval
- CIES Carrier Induced Epitopic Suppression
- CL Constant Light Chain Domain of immunoglobulin
- CP Classical Pathway.
- CR Complement Receptor
- CRM197 Non-toxic Mutant of Diphtheria Toxin
- CSF Cerebrospinal Fluid
- CSR Class Switch Recombination
- CV Column Volume.
- D Diversity Region
- Da Daltons
- DAF Decay-Accelerating Factor
- DAMPS Damage Associated Molecular Patterns
- DC Dendritic Cell
- DIC Disseminated Intravascular Coagulation
- DNA Deoxyribose Nucleic Acid
- DT Diphtheria Toxoid
- ECL Electrochemiluminescence
- EDC 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride.
- EDTA Ethylenediaminetetraacetic Acid.
- EGTA ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid.
- ELISA Enzyme-Linked Immunosorbent Assay
- Fab Antigen Binding Fragment of Immunoglobulin
- FB Factor B
- Fc Crystallisable Fragment of Immunoglobulin
- FcR Fc Receptor

- FD Factor D
- FH Factor H
- FHbp Factor H binding protein
- FHR Factor H Related Protein
- FI Factor I
- FITC Fluorescein Isothiocyanate
- FPLC Fast Protein Liquid Chromatography
- GAG Glycosaminoglycan
- GMC Geometric Mean Concentration
- GPI Glycosylphosphatidyl Inositol
- GSK GlaxoSmithKline
- h/rSBA Serum Bactericidal Assay performed with human/rabbit serum
- HBS HEPES buffer solution
- HBSS Hank's balanced salt solution
- HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
- HI Heat Inactivated
- HL60 Human promyelocytic leukaemia cell line 60
- HRP Horse Radish Peroxidase
- ICP Intracranial Pressure
- IFN-γ Interferon Gamma
- IgG/M Immunoglobulin G/M
- IL Interleukin
- J Junctional Region
- KD Dissociation constant
- LC Light Chain of Immunoglobulin
- LPS Lipopolysaccharide
- mAb monoclonal Antibody.
- MAC Membrane Attack Complex
- MASP MBL Associated Serine Protease
- mAU milli Absorption Units.
- MBL Mannose-Binding Lectin
- MCP Membrane Cofactor Protein
- MenACWY Meningococcal polysaccharides from serogroups A, C, W and Y
- MFI Mean Fluorescence Index.
- MHC Major Histocompatibility Complex
- mHSA methylated Human Serum Albumin.
- LP Lectin Pathway.
- MwM Molecular weight Marker
- n number of samples
- NK Natural Killer
- NR Non-Reduced
- OPD o-Phenylenediamine dihydrochloride.
- PAMP Pathogen Associated Molecular Patterns
- PBS Phosphate Buffered Saline
- PCR Polymerase Chain Reaction

- PorA Porin A
- R Reduced
- Rmax maximum analyte binding capacity of the surface in RU
- RPM Rotations Per Minute.
- RU Response Units
- SBA Serum Bactericidal Assay
- SCR Short Consensus Repeat
- SDS-PAGE Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
- serpin serine protease inhibitor
- SPR Surface Plasmon Resonance
- TD T Cell-Dependent Antigen
- TGF-β Transforming Growth Factor Beta
- TI T Cell-Independent Antigen
- TMB Tetramethylbenzidine.
- TT Tetanus Toxoid
- V Variable Region of immunoglobulin
- v/v volume/volume
- VBS Veronal Buffered Saline.
- w/v weight/volume.
- WHO World Health Organisation

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Chapter One – Introduction

1.1 *Neisseria meningitidis*

Neisseria meningitidis is a species of Gram-negative, semi-commensal, diplococcus bacterium that can cause pathology in the nervous and circulatory systems of humans. Invasive infection can result in rapidly developing, life-threatening meningitis and meningococcemia. Serogroups of *Neisseria meningitidis* are categorised by different polysaccharides expressed on their outermost membrane. To date, 13 serogroups have been identified with A, B, C, W-135, X and Y responsible for the majority of infections throughout the world (Halperin et al., 2011). These serogroup-specific, carbohydrate structures are the antigens for many current meningococcal vaccines.

Meningococcal vaccines are one of the most effective methods for the prevention of disease. Several correlates of protection have been developed to measure the efficacy of vaccination. Each is designed to assess the function of antibodies induced by vaccination in the clearance of *Neisseria meningitidis*. The serum bactericidal assay (SBA) measures the ability of antibodies in serum to kill *Neisseria meningitidis* through complement dependent cytotoxicity (CDC) (Goldschneider et al., 1969a; Goldschneider et al., 1969b; Maslanka et al., 1997). The dilution factor of an individual's serum inhibiting 50% of bacterial growth is known as the SBA titre and titres greater than four are considered protective (Borrow et al., 2005). The SBA is the gold standard for the approval of new meningococcal vaccines (WHO, 1976, 2004, 2006).

The SBA requires a source of complement from an individual lacking any pre-existing bactericidal activity towards each serogroup of bacteria being tested. High carriage rates and vaccination programmes often render finding a human donor of complement for SBAs fruitless

and standardisation difficult. As a result, baby rabbit serum (BRS) is recommended as the complement source to measure meningococcal vaccine efficacy (Maslanka et al., 1997).

When a human donor is available, and comparisons between rabbit and human complement in SBAs have been made, titres correlate poorly questioning the validity of using rabbit serum as the source of complement (Findlow et al., 2009; Gill et al., 2011b). Variations in the ability of human antibody subclasses to activate human and rabbit complement and species-specific differences in the interaction of complement regulatory proteins and *Neisseria meningitidis* are thought to be responsible for the poor correlation between SBAs with human (hSBA) and rabbit (rSBA) serum (Del Tordello et al., 2014; Gill et al., 2011b; Lewis et al., 2013; Santos et al., 2001; Zollinger and Mandrell, 1983). A much better understanding of the SBA and how titres correlate with protection against invasive disease is needed.

1.1.1 Structure and Anatomy of Neisseria meningitidis

As previously stated, *Neisseria meningitidis* can be described as a Gram-negative, diplococcus bacterium. The descriptor 'diplococcus' refers to the spherical appearance of *Neisseria meningitidis* and that the bacterium typically forms pairs. Diplococcus is derived from the Greek *'diploos'* meaning double and *'kokkos'* meaning berry. The descriptor 'Gram-negative' refers to the fact that meningococci appear red (or pink) when Gram-stained as a result of their inability to retain the crystal violet dye used in this process. The counterstain safranin, which is also employed in this process, is responsible for the red colouring of the bacterium. Bacteria that retain the crystal violet dye and appear purple are known described as 'Gram-positive'.

The technique of crystal violet dye to stain bacteria was developed by and subsequently named after the Danish bacteriologist Hans Christian Gram (1853–1938) in 1884 (Gram and Friedlaender, 1884). First devised as a method to make bacteria more visible in lung sections

from deceased pneumonia patients, Gram-staining is a commonly used technique to distinguish one type of bacteria from another and more specifically, the composition of their cell wall (Beveridge, 2001).

Typically, the cell wall of Gram-positive bacteria consists of a single lipid membrane surrounded by a thick peptidoglycan layer containing both lipoteichoic acid and teichoic acid. The cell wall of Gram-negative bacteria consists of a thin peptidoglycan layer contained within the periplasmic space which are sandwiched between an inner and outer lipid membrane (Beveridge, 1999; Shockman and Barren, 1983). This difference in the thickness of the peptidoglycan layer and the presence/composition of the outer lipid membrane of Gram-negative bacteria is responsible for the ability of Gram-negative bacteria to leach the crystal violet dye while Gram-positive bacteria retain the crystal violet dye in the presence of ethanol (Salton, 1963). Additionally, the outer lipid membrane of Gram-negative bacteria often contains lipopolysaccharides, porins and other proteins (Beveridge, 1999).

As a Gram-negative bacterium, the outer lipid membrane of *Neisseria meningitidis* contains porins (such as PorA), lipoproteins (such as FHbp) and lipopolysaccharide, which can be released into the immediate environment by the bacteria in the form of 'blebs' (Devoe and Gilchrist, 1973; Jarva et al., 2005; Schneider et al., 2009). Often (and particularly by invasive isolates), *Neisseria meningitidis* can also express a polysaccharide capsule, which is antigenically unique to each serogroup and forms the basis of most current meningococcal vaccines (Armand et al., 1982; Artenstein et al., 1970; Gotschlich et al., 1972; Griffiss et al., 1981; Hankins et al., 1982). Meningococcal polysaccharides are either homopolymer composed of mannosamine phosphate (serogroup A), glucosamine phosphate (serogroup X) or sialic acid (serogroup B and serogroup C) or heteropolymers composed of sialic acid with glucose (serogroup Y) or galactose (serogroup W135) (Bhattacharjee et al., 1975; Bundle et al., 1974; Lamb et al., 2005; Moore et al., 2007). Due to the similarities in structure of the polysaccharide capsules expressed by the

serogroups W135 and Y, cross-reactivity of serogroup-specific antibodies between these two serogroups has been shown (Reyes et al., 2013). The composition and size of these meningococcal polysaccharides have been shown to impact on their immunogenicity (Artenstein et al., 1970; Gotschlich et al., 1970; Gotschlich et al., 1972).

1.1.2 Meningococcal Disease

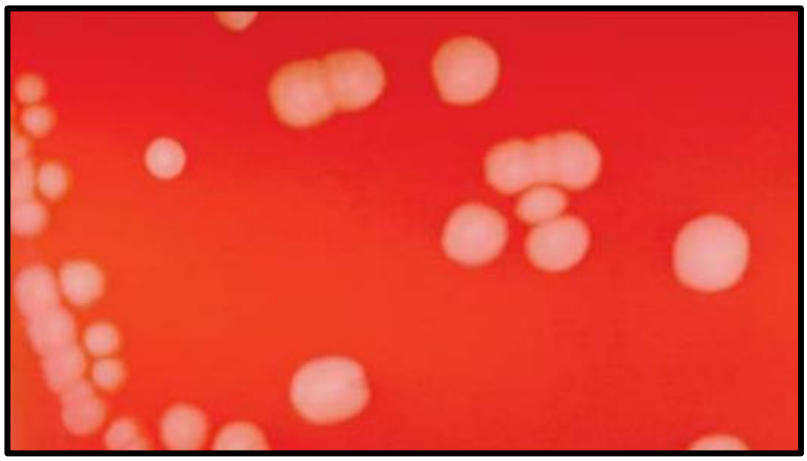
Neisseria meningitidis populates the mucosal lining of the nasopharynx of the host, often without causing noticeable pathology (Rake, 1934). Binding is mediated by type IV pili extruding from the surface of the bacterial body that express specificity for CD147 (Bernard et al., 2014). The major pilin PilE and the minor pilin PilV both interact directly with the second immunoglobulin domain of CD147. *Neisserial* type IV pili also bind and signal through the complement regulator membrane co-factor protein (MCP or CD46) and β2-adrenergic receptor (Coureuil et al., 2010; Källström et al., 1998). Carriage rates are high and range from 6% in infants to 25% in 18-19-year-olds after which rates decline to 8% in those aged 50 years and older (Christensen et al., 2010). *Neisseria meningitidis* is transmitted from person-to-person through respiratory and throat secretions.

Meningitis is the inflammation of the meninges; a collective term for the dura mater, arachnoid, and pia mater membranes that surround the brain and spinal column. Once in the circulation, the bacterium colonises the meninges and cerebral spinal fluid (CSF) of the host. Subsequent inflammation increases the permeability of the blood-brain barrier (BBB) allowing entry of immune cells to combat the infection. The release of potent inflammatory cytokines by invading leukocytes and neutrophils into the CSF leads to an increase in intracranial pressure and ischemia (Sáez-Llorens and McCracken, 2003).

Typically, patients present with headaches, nausea, neck stiffness and fever (van de Beek et al., 2004). Only 22% of patients present with the characteristic purpura often associated with the disease. This purpura manifests from endotoxin release from lysed *Neisseria meningitidis* which activate clotting factors in the circulation leading to disseminated intravascular coagulation (DIC) (Colman et al., 1972). The resulting occlusion of peripheral blood vessels and subsequent ischemic tissue damage of skin and limbs may result in extensive scarring and amputation of the affected limb. Furthermore, the rapid depletion of circulating platelets and clotting factors increases the risk of haemorrhage (Levi and Ten Cate, 1999).

If left unresolved, meningitis can lead to cardio-respiratory failure, seizures and death. Case fatality rates (CFR) for meningococcal disease average between 9-12% but can be as high as 50% with the addition of septicaemia. Around 20-25% of those patients who survive will develop a long-lasting morbidity. These morbidities commonly include seizures and epilepsy, the loss of hearing and sight, limb loss, and learning and behavioural difficulties in children (Sáez-Llorens and McCracken, 2003; Van de Beek et al., 2006). *Neisseria meningitidis* infection is diagnosed by examination and bacterial culture of CSF or blood, although polymerase chain reaction (PCR) is now more commonly used (Gray et al., 2006; Stephens et al., 2007). PCR is often the preferred method of diagnosis as CSF cultures may provide false negative results if antibiotics are administered before testing (Kristiansen et al., 1991). CSF is aspirated by lumbar puncture between the vertebrae L3/L4 or L4/L5. CSF or blood samples from patients with suspected disease are cultured on blood agar and Gram-stained for identification. Positive cultures are seen as round, moist, Gram-negative, convex colonies (***Figure 1. 1***) (Gray and Fedorko, 1992).

a



b

Figure 1. 1 *Neisseria meningitidis* Colonies on Agar Plates
Colonies of *Neisseria meningitidis* on blood agar (***a***) and chocolate agar (***b***) plates. Images taken from Laboratory Methods for the Diagnosis of Meningitis, Chapter 7: Identification and Characterization of *Neisseria meningitidis* published by Centres for Disease Control and Prevention. Website address: http://www.cdc.gov/meningitis/lab-manual/chpt07-id-characterization-nm.html.
Accessed 29/08/2016.

1.1.3 Epidemiology

Globally, the prevalence of each meningococcal serogroup varies significantly between countries and regions, with serogroups A, B, C and Y responsible for the majority of reported cases (Harrison et al., 2009; Lingani et al., 2015; Vyse et al., 2011). A summary table of disease rates and distribution of serogroups by region is shown in ***Table 1. 1***. The greatest burden of disease occurs in Sub-Saharan Africa in an area known as the African Meningitis Belt. The African Meningitis Belt is formed of 18 countries with the majority of cases occurring in Burkina Faso, Chad, Ethiopia, and Niger. Unlike most regions where B, C and Y are the most commonly found serogroup, serogroup A is predominant in the African Meningitidis Belt (roughly 60% of all reported cases). The Meningitis Vaccine Project, created through the collaboration of The World Health Organisation and Program for Appropriate Technology in Health (PATH), is currently running a mass vaccination programme in an attempt to combat serogroup A disease in the region. Early reports suggest a significant reduction in rates of invasive serogroup A meningococcal disease (Lingani et al., 2015).

In the UK, predominant serogroups include B, C W and Y with serogroup B accounting for more than 60% of all infections. Routine vaccination against serogroup C was introduced to the UK in 1999 following a significant increase in reported infections. Vaccination has been very successful with only 28 serogroup C cases reported in 2014/2015 compared to 883 in 1998/1999. Recently, vaccines against serogroups B and A, C, W, Y were introduced into the vaccine schedule to reduce the large numbers of serogroup B infections and counter the worrying spike in serogroup W infections (Lucidarme et al., 2015). In Europe, case fatality rates remain between 5-10% for ages 0-44 years of age increasing to 20% of those aged >65. The variation in rates of meningococcal disease in the UK by year and age are shown in ***Figure 1. 2***.

Table 1. 1 Worldwide Rates of Meningococcal Disease and Distribution of Serogroups

Global incidence rates (per 100,000 people), case fatality rates (%) and distribution of serogroups (percentage of total isolates) for meningococcal disease.

Region	Incidence Rate (per 100,000 population)	Case Fatality Rate (%)	Year of Study	Distribution of Serogroups (%)						
				A	*B*	*C*	*W*	*Y*	*X*	*Other*
Australia	1.5	3.4	2005-2007	-	83.5	8.3	-	-	-	8.2
South Africa	0.64	No Data	1999-2002	23	41	8	5	21	-	2
New Zealand	2.3	5.9	2009-2013	-	52.6	29.8	8.8	7	-	2
United States	0.53	11.3	1998-2007	-	29.9	28.8	2.5	34.8	-	4
Japan	0.01	No Data	1990-2003	-	57	-	1	21	-	21
Europe	1.44	7.75	1999-2004	0.7	75.4	17.6	2.7	2.2	1.3	0.1
China	0.09	9.95	2005-2010	36.8	11.4	43.3	0.4	-	-	8.1
Canada	0.67	9.01	2002-2003	-	41.8	36.4	3.2	17.7	-	0.9
Brazil	1.9	20	2006	-	38.2	55.2	5	1.6	-	-
Argentina	0.7	10	2007	-	68.8	11.4	13	6.8	-	-
Mexico	0.06	No Data	2000-2005	-	12	71	-	8	-	9
African Meningitis Belt	13.6	10.3	2004-2009	73.5	-	-	13.6	2.8	8.2	1.9
	6.4	9.5	2010-2013	1.6	-	-	38.4	-	59.3	0.8

Table References: (Baethgen et al., 2008; Caugant et al., 2012; Chiu et al., 2010; Coulson et al., 2007; Dickinson and Pérez, 2005; Harrison, 2006; Harrison et al., 2009; Ilyina et al., 2014; Laan et al., 2000; Li et al., 2015; Li, 2014; Lingani et al., 2015; Martin et al., 2007; Network, 2006; Rosenstein et al., 1999; Sáfadi and Cintra, 2010; Takahashi et al., 2004; Vyse et al., 2011; Watkins et al., 2006)

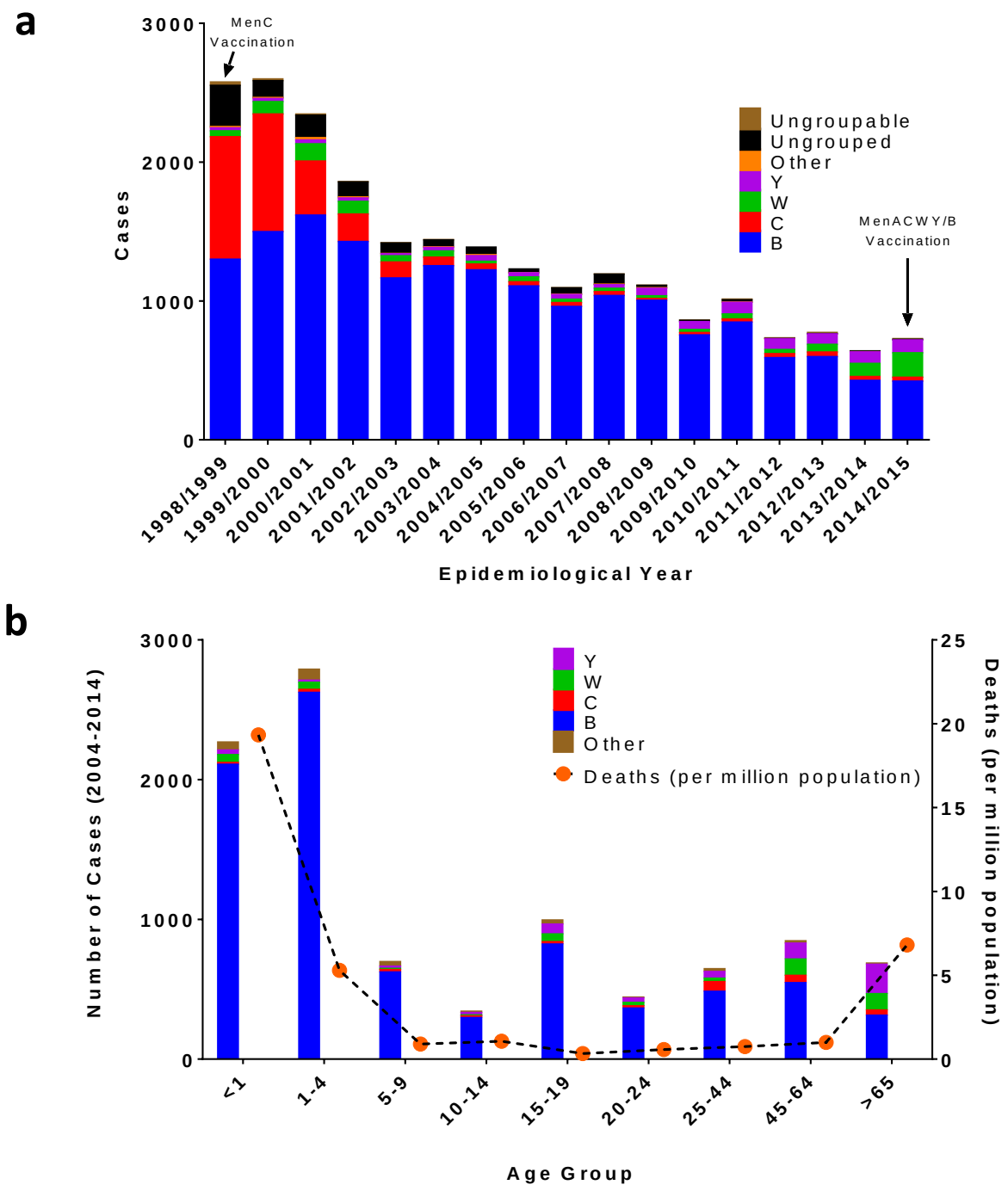


Figure 1. 2 Cases of invasive *Neisseria Meningitidis* Infection in the UK

a, Epidemiological data of confirmed invasive *Neisseria Meningitidis* infection by serogroup for epidemiological years 1998/1990 to 2014/2015. ***b***, Cases and deaths per million population of invasive *Neisseria Meningitidis* infection by age for each serogroup between 2004 and 2014. Data collected from the Office of National Statistics and Public Health England.

1.1.4 Treatment of Disease

Meningococcal disease is classified by either the presence or absence of meningitis and/or meningococcemia (Feavers et al., 2015; McGill et al., 2016). If meningococcal disease is suspected immediate intravenous antibiotics (such as cefotaxime or ceftriaxone) and steroids (such as dexamethasone) are recommended to stem any further bacterial growth and reduce the symptoms of shock (Visintin et al., 2010). If meningitis and meningococcemia are both present, treatment of the meningococcemia will take priority due to its association with a much poorer prognosis (Van Deuren et al., 2000).

Dissemination of *Neisseria meningitidis* infection into the bloodstream (meningococcemia) is often accompanied by septic shock and DIC. Intravenous fluid, inotropic drugs and oxygen are administered to maintain blood pressure, tissue perfusion and oxygen saturation (Carcillo et al., 1991). Mass consumption of clotting agents related to DIC is treated with vitamin K, plasma and platelet infusion to prevent bleeding and ischemia (Qu et al., 2011).

Colonisation of the meninges by *Neisseria meningitidis* precedes meningitis (cerebral oedema) and is associated with increased intracranial pressure (ICP). Increased ICP can be combatted with intravenous infusion of either a saline or sugar solution (Wakai et al., 2013). However, it must be noted that the benefit of mannitol therapy in the treatment of increased ICP is under question and may even worsen ICP. All efforts are made to reduce any exacerbations in ICP by sedating patients, keeping patients cool and in an upright position and active prevention of seizures.

1.1.5 Meningococcal Vaccines

Vaccination aims to produce immunological memory to a pathogen and to protect the host from disease if exposed to the same pathogen in the future. This can be achieved by introducing an antigen into the host initiating an adaptive immune response specific to the antigen (Boyd, 1946; Clem, 2011). An adequate immune response will activate both humoral and cell-mediated immunity on subsequent exposure. Long lasting memory B and T cells as well as circulating antibodies all provide a quick protective response if exposed to the pathogen post immunisation.

The phenomenon of generating immunity to a disease by vaccination can be traced to Edward Jenner in his classical papers on *Variolæ vaccinæ* and smallpox (Jenner, 1801). Edward Jenner made the observation that individuals who had previously suffered from vaccinia virus infection were subsequently protected from smallpox. This observation eventually led to the production of an effective smallpox vaccine and successful eradication of the disease in 1969 (Fenner et al., 1988).

Vaccine antigens are generated and administered in many forms (Artenstein et al., 1970; Control, 1989; Donnelly et al., 1996; Sabin, 1985). Vaccines can comprise either dead or live attenuated pathogen (polio and measles vaccines, respectively), a subunit of a pathogen, a toxoid (tetanus vaccines), polysaccharides taken from the outer shell of the bacterial wall (meningococcal vaccines) or the DNA taken from the pathogen itself. The desired response to meningococcal vaccines is the production of high titres of protective antibodies.

Much of today's understanding of the protection against meningococcal disease and the effectiveness of capsular polysaccharides as a vaccine antigen was presented in a series of seminal papers entitled 'Human Immunity to the Meningococcus I-V' published in 1969 by Malcolm S. Artenstein, Emil C. Gotschlich and Irving Goldschneider of the Walter Reed Army Institute of Research, Washington. Artenstein et al., showed that in most cases the susceptibility

to meningococcal disease is related to a lack of meningococcal-specific bactericidal antibody and that antibody specific to the polysaccharide capsule of *Neisseria meningitidis* form a major part of the bactericidal titres of sera (Goldschneider et al., 1969a; Goldschneider et al., 1969b). Artenstein et al., also showed that natural immunity to the meningococcus could be acquired through either asymptomatic carriage of meningococci in the nasopharynx or due to transplacental passage of meningococcal-specific IgG antibodies from mother to foetus and that bactericidal antibodies could be induced by vaccination with the capsular polysaccharide of *Neisseria meningitidis* (Goldschneider et al., 1969b; Gotschlich et al., 1969c). The first vaccines were developed against serogroups A and C in the 1960s and 70s following the successful purification of high molecular weight (>100kDa) polysaccharides from the bacteria (Gotschlich et al., 1969a; Gotschlich et al., 1969c). These preparations induced significant bactericidal titres in humans; a vast improvement on previous studies using low molecular weight (<50kDa) (Gotschlich et al., 1969a, b; Kabat et al., 1944). Further investigations on the immunogenicity of these high molecular weight polysaccharides showed that responses are serogroup-specific and not cross-reactive to other serogroups, and that vaccination can reduce serogroup-specific nasopharyngeal acquisition but not the overall carriage of meningococci due to replacement by other serogroups (Gotschlich et al., 1969a, b; Gotschlich et al., 1969c). Soon after, vaccines composed of the polysaccharides isolated from serogroups W and Y were introduced (Armand et al., 1982; Griffiss et al., 1981; Hankins et al., 1982).

Until recently, efforts to produce a protective serogroup B vaccine by the same process have been unsuccessful due to similarities between serogroup B polysaccharides and brain glycoproteins (Finne et al., 1983; Wyle et al., 1972). The genome of a serogroup B isolate was sequenced in the hope to identify alternative antigens suitable for vaccination (Tettelin et al., 2000). Genes coding for 570 potential vaccine antigens were identified. A total of 330 of these were successfully expressed in *Escherichia coli* and used to immunise mice in the goal of

determining those antigens most appropriate for vaccination (Pizza et al., 2000). Seven of these antigens were shown to be highly expressed by *Neisseria meningitidis* and able to generate bactericidal antibody in mice. Individually, these antigens did not produce bactericidal antibody to all strains tested due to inter-strain variability of these antigens (Giuliani et al., 2006); however, a combination of five of these antigens did generate a broad bactericidal response and now form the basis of one of the new serogroup B vaccines, Bexsero™ (Gorringe and Pajon, 2012). It was later discovered that one of these antigens specifically binds the soluble complement regulator factor H (FH), now known as factor H binding protein (FHbp) (Madico et al., 2006). Another serogroup B vaccine, composed of two FHbp subfamily members, is in development (Jiang et al., 2010).

Characteristic of T cell-independent B cell activation, responses to meningococcal polysaccharide vaccines are weakly immunogenic and short-lived (Gold et al., 1975). To improve the immunogenicity of meningococcal vaccines in young children, polysaccharide antigens are often covalently linked to protein conjugates (Costantino et al., 1992; Findlow and Borrow, 2016; Pichichero, 2005). Common conjugates include tetanus toxoid (TT), non-toxic mutant of diphtheria toxin (CRM197) and diphtheria toxoid (DT). Protein conjugates of meningococcal polysaccharides show superiority over plain polysaccharides by inducing immunological memory and higher SBA titres (Leach et al., 1997; Li et al., 2014; Richmond et al., 1999a). Conjugate proteins have the potential to both boost or suppress the immune response to polysaccharide antigens (Findlow and Borrow, 2016). The process where prior immunity to a conjugate protein decreases the immune response to a polysaccharide antigen conjugated to the same protein is known as carrier induced epitopic suppression (CIES). CIES has been described for a TT-conjugated MenC vaccine when administered to individuals previously vaccinated with another vaccine containing TT (Burrage et al., 2002). The exact mechanism behind CIES is unknown but thought to occur when either antibody specific to the conjugate protein sterically hinder B cell

access to the polysaccharide antigen, when the immune response to the carrier protein is prioritised over the response to the polysaccharide antigen and/or when conjugate protein-specific regulatory T cells dampen the response to the polysaccharide antigen.

1.1.6 Correlates of Protection

Neisseria meningitidis infection is cleared by both CDC and phagocytosis (Granoff, 2009; Lo et al., 2009). Deficiencies in opsonins and complement proteins are associated with increased susceptibility to invasive meningococcal disease (Bathum et al., 2006; Hellerud et al., 2010). These mechanisms of protection are enhanced by bacteria-specific antibody acquired either by natural exposure to *Neisseria meningitidis*, vaccination or passively by placental transfer (Goldschneider et al., 1969a; Goldschneider et al., 1969b; Gotschlich et al., 1969a, b). Correlates of protection examine these mechanisms of immunity to assess whether an individual is protected against a specific pathogen. Several correlates of protection exist for *Neisseria meningitidis* which are used to evaluate the efficacy of meningococcal vaccination, including the SBA, opsonophagocytic assay and ELISAs to measure the concentration of anti-meningococcal antibody (Borrow et al., 2005; Domnich et al., 2015; Humphries et al., 2015). The SBA is the gold standard correlate of protection used for the approval of new meningococcal vaccines and will be the main focus of this project.

1.1.5.1 Serum Bactericidal Assay

The SBA is a standard laboratory technique used to measure the efficacy of meningococcal vaccines and is a suitable measurement for regulatory approval (Maslanka et al., 1997). The SBA measures the bactericidal activity of an individual's serum towards *Neisseria meningitidis*. Briefly, serum from an individual is heat inactivated and serially diluted before mixing 1:1 with a source of active complement. Heat inactivation of the test sample removes complement activity without significantly altering the level of antibody. Sources of complement include human serum and BRS. Live *Neisseria meningitidis* bacteria, of a specific serogroup, is then added to this mixture, plated out and incubated overnight. The number of colonies is then counted and compared to the negative control (no sample present and heat-inactivated complement source). The dilution of the test sample where 50% bacterial growth is inhibited is known as the SBA titre. Susceptibility to meningococcal disease significantly correlates with SBA titre and titres greater than four to eight are considered protective (Borrow et al., 2001a; Borrow et al., 2005; Goldschneider et al., 1969a; Goldschneider et al., 1969b; Gotschlich et al., 1969a; Rezaei et al., 2007).

1.2 Immunoglobulins

Antibodies are a secreted form of the B cell receptor (BCR). The very first description of an antibody [fragment] has been attributed to Dr Henry Bence Jones; while examining the urine of a patient suffering from multiple myeloma in 1848 he noticed that heating the urine provoked a precipitate (Jones, 1848). Named after its originator, the Bence-Jones protein has since undergone extensive analysis and been identified as the disassociated light chain of an antibody (Delman and Gally, 1962). However, the first real description of antibodies is credited to Emil von Behring and Shibasaburo Kitasato in 1890 (Von Stabsarzt, 1890). Behring and Kitasato discovered a substance in serum able to neutralise tetanus toxin and convey immunity to

disease. This substance could be transferred in serum from immune animals into non-immune animals, generating passive protection against disease. This soluble serum protein with antitoxin properties was first referred to as antibody or 'antikörper' by Paul Ehrlich in 1891, although this term was not used commonly until several years later (Ehrlich, 1891; Lindenmann, 1984).

In humans, antibodies can be separated into five distinct classes: IgG, IgM, IgA, IgD and IgE (Black, 1997). Initially known as the 7S γ-globulin (or variations thereof) as a result of sedimentation and mobility studies, IgG was first discovered and is the most studied of the immunoglobulins (Cohen, 1965; Tiselius, 1937; Tiselius and Kabat, 1939). The immunoglobulin IgM was identified soon after; followed by IgA, IgD and finally IgE (Grabar and Williams, 1953; Heremans et al., 1959; Ishizaka et al., 1966; Kabat, 1939; Rowe and Fahey, 1965; Waldenström, 1944). The nature of B cell activation dictates the subclass of antibody secreted into the circulation (Nutt et al., 2015).

1.2.1 Antibody Structure

The core antibody unit is formed of four chains (two heavy chains and two light chains) held together with multiple disulphide bonds. Studies by Rodney Porter in the 1950s and 1960s on the antigenic similarities and functions of IgG fractions, generated through papain digestion and reduction of the disulphide bonds, led to the basic model of antibody structure that is still used today (Porter, 1962, 1963) (***Figure 1. 3***).

Papain digestion of IgG produces three fractions, first assigned the names I, II and III based on their mobility through a carboxymethylcellulose matrix (Petermann, 1946; Porter, 1950, 1958, 1959). Fractions I and II both bind antigen and inhibit antigen precipitation by the intact antibody. Fraction III does not bind antigen but does fix complement (Ishizaka and Campbell, 1958; Ishizaka et al., 1962). These factions were later termed Fab and Fc based on their antigen-binding (ab) function and tendency to crystallise (c) out of solution (Cohen, 1965).

Reduction of the disulphide bonds that holds IgG together with β-mercaptoethylamine and urea produced two sub-units of differing molecular weights (Edelman and Poulik, 1961; Edelman, 1959). Based on their respective molecular weights (50kDa and 25kDa), these sub-units were termed heavy (H) and light (L) chains of antibody (Cohen, 1965; Pain, 1963). Two H chains and two L chains form a complete IgG molecule at a size of roughly 150kDa (Kabat, 1939). Immunisation of rabbits with the different subunits and fractions of human IgG and subsequent investigations on the cross-reactivity of the resultant antisera with these components showed that the H chain contained elements of both Fab and Fc fragments and the L chain contained only elements of the Fab fragment (Cohen, 1963). These data along with chemical composition investigations led to the well-known basic primary protein structure of IgG as shown in ***Figure 1. 3*** (Porter, 1962).

The first complete amino acid sequence of an IgG[1] molecule was published in 1969 by Gerald M. Edelman et al., revealing several regions of homology throughout the heavy and light chains (Edelman et al., 1969). These finding supported the hypothesis that both the heavy and light chains of immunoglobulins are split into two and four domains, respectively. These domains are known as the variable light chain (VL) and constant light (CL) chain domains of the light chain and variable heavy chain (VH), CH1, CH2 and CH3 domains of the heavy chain. In 1968, the first x-ray crystallography structure of an intact IgG molecule (hinge deletion IgG1 'Dob' antibody) was achieved (Terry, 1968), enabling the solution of the complete quaternary structure of this IgG1 antibody and confirming previous predictions of the multi-chain, multi-domain arrangement of IgG (Sarma et al., 1971; Silverton et al., 1977; Terry, 1968).

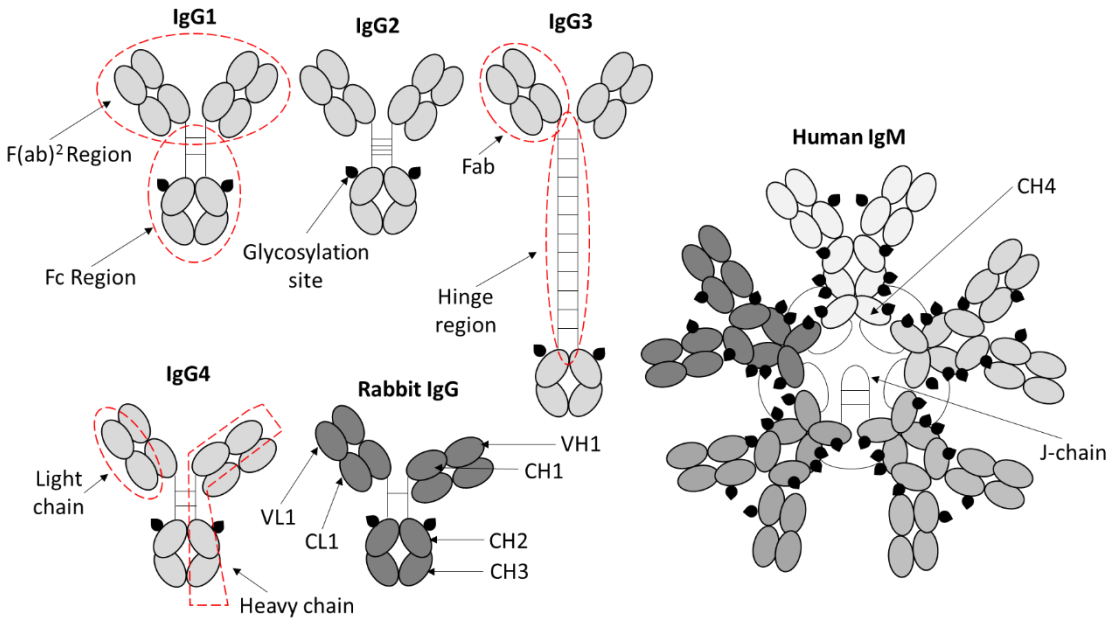


Figure 1. 3 Immunoglobulin Structure

The core antibody unit consists of two heavy and two light chains held together with multiple disulphide bonds. IgM is formed of five of these antibody units. The heavy and light chains are split into several domains: variable light chain domain (VL), constant light chain domain (CL), variable heavy chain domain (VH) and constant heavy chain domains (CH). The CH1 and CH2 domains are connected via the hinge region, which varies in length between the subclasses. Papain digestion of the hinge region splits antibody into two fragments: Fc and Fab regions. A glycosylation site is contained within each CH2 domain of IgG antibody. Each IgM antibody unit has ten glycosylation sites: one on each CH1 domain, one on each CH2 domain, two on each CH3 domain and one on each CH4 domain.

1.2.2 IgG Subclasses

The IgG class of human antibody can be further divided into four subclasses: IgG1, IgG2, IgG3 and IgG4. These were first identified due to antigenic variation of their heavy chains (Grey and Kunkel, 1964; Terry and Fahey, 1964). The IgG subclasses were named in accordance to their abundance in plasma with IgG1 (66% of total IgG) the most and IgG4 (2%) the least abundant (French and Harrison, 1984; Kunkel et al., 1965). The IgG subclasses are similar in size (146 to 170kDa) and share roughly 92-96% sequence similarity (Hamilton, 1987). Differences between the IgG subclasses are shown in ***Figure 1. 3*** and ***Table 1.2***. The most striking difference between the structure of IgG subclasses is the relative length of the hinge region that joins the CH1 and CH2 domains (Gregory et al., 1987; Hamilton, 1987). IgG3 possesses the longest hinge region at 62 residues followed by IgG1 at 15 residues, IgG2 and IgG4 at 12 residues each (Edelman et al., 1969; Michaelsen et al., 1977; Vidarsson et al., 2014). IgG subclasses significantly differ in their ability to activate complement and their affinities to Fc receptors.

1.2.3 Antibody Function

Antibodies express multiple effector functions once bound to their antigen. These functions result directly from or indirectly from binding antigen. As a consequence of differences in structure and composition, each subclass of antibody exhibits significant variation in their function.

Table 1. 2 Properties of Immunoglobulins

Several structural and functional properties of rabbit IgG, human IgM and human IgG subclasses are shown. Rabbit immunoglobulin only consists of one subclass. The cytokines and antigens responsible for the upregulation or inhibition of each antibody subclass are also summarised.

Property		Antibody					
		IgG1	**IgG2**	**IgG3**	**IgG4**	**IgM**	**IgG**
Species		Human	Human	Human	Human	Human	Rabbit
Serum Concentration (mg/ml)		8	4	0.8	0.4	1.3	13.6
Complement Fixation		++	+	+++	-	+++	++
Hinge Length (aa)		15	12	62	12	N/A	11
Size (kDa)		146	146	170	146	970	144
Specificity for	**Protein**	++	+/-	++	+/-	+	+
	Polysaccharide	+	++	-	-	+	+
	Allergens	+	-	-	++	-	+
Isoelectric Point		8.6±0.4	7.4±0.6	8.3±0.7	7.2±0.8	5.5 - 7.4	6.1–6.5
Stimulatory Cytokines		IL-4; IL-10	IFN-γ; TGF-β	IFN-γ; IL-10	IL-4; IL-13; IL-10	IL-2; IL-5	N/A
Inhibitory Cytokines		IFN-γ	IL-4	TGF-β; IL-4	IFN-γ	IL-4; IFN-γ; TGF-β	N/A

Antibodies can neutralise and aggregate pathogens and toxins reducing pathology and enhancing their clearance. The bivalency of an antibody means an antibody molecule can bind two antigens co-currently. Antigens with several epitopes allow the binding of multiple antibodies, each able to bind two antigens, creating an ever-growing cross-linked lattice structure (Dean and Webb, 1926). In this way, pathogens or toxins are concentrated, enhancing complement activation and increasing the speed and efficiency of clearance by phagocytes (Margni et al., 1980; Perdigón et al., 1982; Wright et al., 1980). Neutralising antibodies inhibit the biological actions of its antigen whether by preventing the transportation of a pathogen across a membrane or by directly blocking the actions of toxins (Davis et al., 1978). Introducing neutralising antibody to the meningococcal endotoxin LPS, which is responsible for the development of disseminated intravascular coagulation, into patients suffering from meningococcemia significantly reduced mortality rates (Ziegler et al., 1982). All antibody subclasses can aggregate and neutralise antigens.

The Fc region of antibody bound to an antigen can activate complement, enhance phagocytosis and activate effector cells of the immune system, each activity aiding the clearance of pathogens. The first component of the classical pathway of complement activation, C1q, binds the CH2 domains of IgG and IgM antibody classes; the C1q binding site becomes exposed when the antibody is bound to antigen (Müller-Eberhard and Kunkel, 1961). Subsequent complement activation leads to CDC, opsonisation and chemotaxis. Human IgM and IgG3 antibodies are the best activators of complement followed by IgG1 and IgG2; IgG4 does not activate complement (Brüggemann et al., 1987; Gadjeva et al., 2008; Garred et al., 1989). The presence of complement activating antibody, or bactericidal antibody, and an intact complement system, significantly correlate with the susceptibility to invasive meningococcal disease (Figueroa and Densen, 1991; Goldschneider et al., 1969a; Goldschneider et al., 1969b; Gotschlich et al., 1969a; Granoff, 2009).

Fc receptors (FcRs) bind the Fc region of antibodies and are named according to their specificity (Anderson et al., 1989). For example, FcγRs bind IgG, FcεRs bind IgE and so on. FcRs are expressed on numerous cells types including dendritic cells (DCs), natural killer cells (NK cells) and mast cells (Daëron, 1997). FcR binding leads to either phagocytosis or antibody-dependent cell mediated cytotoxicity (ADCC) of the targeted (opsonised) antigen (Aderem and Underhill, 1999). The IgE-binding FcR, FcεR1, is expressed by mast cells, basophils and eosinophils (Turner and Kinet, 1999). IgE antibody, cross-linked by antigen and bound to FcR, activates mast cells, basophils and eosinophils releasing stored histamine and other inflammatory mediators. In a similar vein, degranulation of NK cells occurs through FcγRIII signalling releasing preformed granules, containing cytotoxic enzymes such as perforin and granzyme A, killing the antibody labelled cell. Activation of FcRs expressed by macrophages and DCs drives phagocytosis of the targeted antigen. Macrophages and DCs don't only help clear infection directly but link innate and adaptive immunity through antigen processing and presentation to cells of adaptive immunity.

1.2.4 B Cell Activation

Antigens able to activate B cells and induce secretion of immunoglobulin are separated into two distinct categories (Mond et al., 1995; Parker, 1993). Those dependent on the help of T cells for activation (TD) and those able to activate B cells in the absence of T cells (TI). The latter can be split further into TI-1 and TI-2 antigens (Vos et al., 2000). TD antigens, such as TT, are proteins able to both bind the B cell receptor (BCR) and engage with major histocompatibility complexes (MHC). TI-1 antigens, such as LPS or bacterial DNA, activate B cells without the help of T cells or engagement of the BCR. TI-2 antigens, such as bacterial polysaccharides, activate B cells by multivalent binding of the BCR (Dintzis et al., 1976). A second stimulatory signal, independent of the original antigen, is usually necessary for the production of secretory

immunoglobulins. This second signal is delivered by the engagement of additional B cell receptors by other immune cells (T cells, NK cells and macrophages), products of complement activation (C3d), damage associated molecular patterns (DAMPS) or pathogen-associated molecular patterns (PAMPS) (Abruzzo and Rowley, 1983; Dempsey et al., 1996; Pasare and Medzhitov, 2005).

1.2.4.1 Antibody Class Switching

Sufficiently stimulated B cells will secrete antigen specific antibody into the circulation of the host (Stavnezer, 1996a). B cells are able to produce antibodies of nine different subclasses (IgM, IgD, IgG3, IgG1, IgA1, IgG2, IgG4, IgE and IgA2) differing primarily in their heavy chain and valency. The constant heavy chain region of an antibody is determined during B cell activation by a process known as class switch recombination (CSR) (Muramatsu et al., 2000). The constant heavy chain locus is made up of each heavy chain gene sandwiched between switch regions (Lee et al., 2001). CSR is driven by the activation-induced cytidine deaminase enzyme (AID) which is significantly upregulated when a B cell is activated (Stavnezer et al., 2008). AID converts cytosine bases to uracil by the process of deamination in and around switch regions. These regions are excised creating double-stranded breaks in the DNA. Double-stranded breaks in the S regions recombine creating a loop of DNA containing the constant heavy chain genes that will be deleted allowing the next constant heavy chain gene in the locus to be transcribed. A simplified model of CSR is shown in ***Figure 1. 4***. Sufficient B cell activation also induces somatic recombination of the variable (V), joining (J) and diversity (D) regions, responsible for the specificity of an antibody, in a process known as affinity maturation (Alt et al., 1992). Affinity maturation selects antibodies with the highest affinities to antigen discarding B cells expressing BCRs with lower affinities.

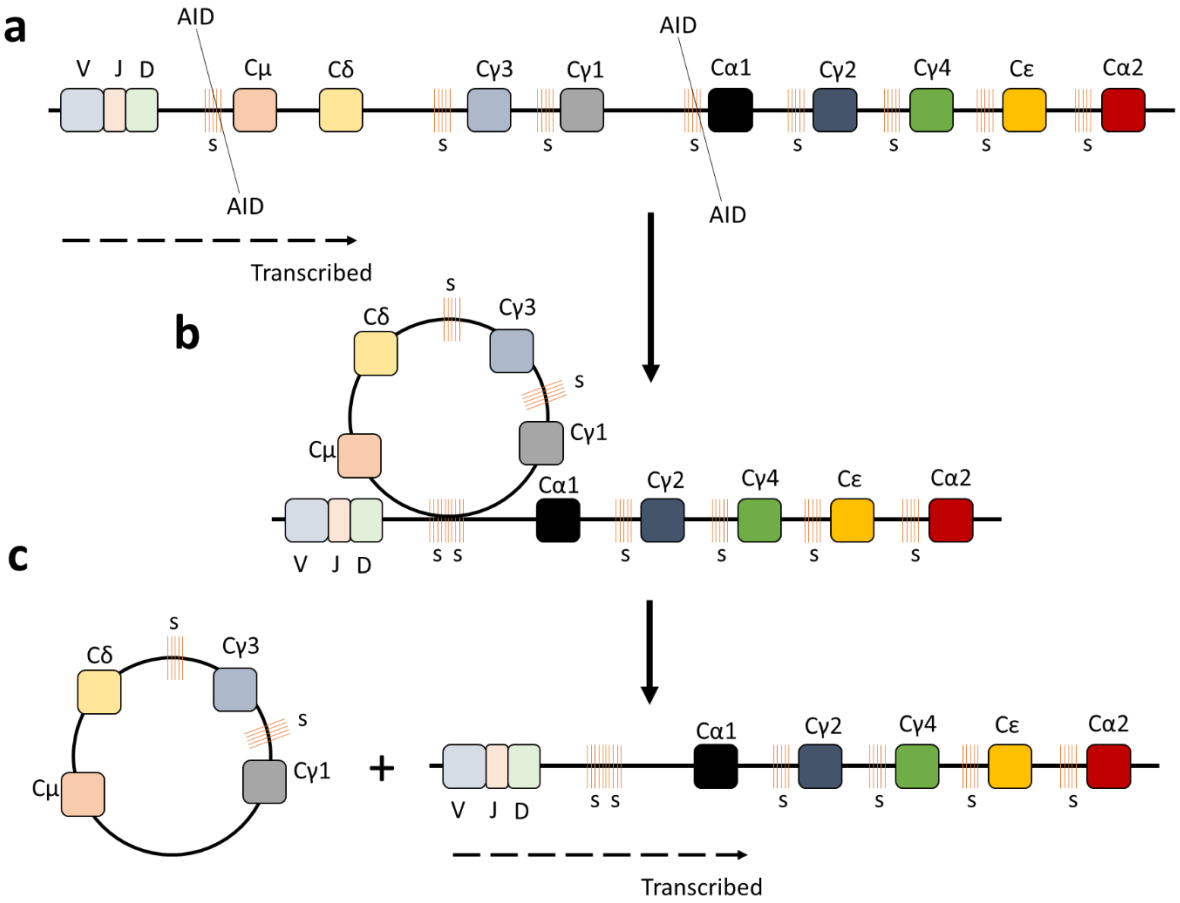


Figure 1. 4 Class Switch Recombination

Class switching of antibodies secreted by activated B cells occurs by a process known as class switch recombination. ***a***, CSR is driven by the activation-induced cytidine deaminase enzyme (AID) which converts cytosine bases to uracil by the process of deamination in and around switch regions (S) flanking genes encoding the constant heavy chains for each class of antibody. Constant heavy chain genes include Cμ (IgM), Cδ (IgD), Cγ1-4 (IgG1-4), Cα1-2 (IgA1-2) and Cε (IgE). ***b***, Deaminated S regions are excised creating double-stranded breaks in the DNA. Double-stranded breaks in the S regions recombine creating a loop of DNA containing the constant heavy chain genes. ***c***, This loop of DNA is then removed allowing the next constant heavy chain gene in the locus to be transcribed. In this case, IgM antibody is class switched to IgA1. Adapted from (Stavnezer et al., 2008).

Cytokines, secreted by helper T cells and other cells of the immune system, directly affects CSR and the class of antibody that is secreted (Kracker and Radbruch, 2004; Stavnezer, 1996b). The initial antibody class secreted in response to an antigen is IgM. Class switching usually occurs within six days post initial activation of the B cell. The cytokines interferon gamma (IFN-γ), transforming growth factor beta (TGF-β), interleukin-4 (IL-4), IL-5, IL-13 and IL-10 each play a crucial role in determining the class of antibody secreted (Coffman et al., 1993). For example, stimulation with IL-4 induces class switching to the IgE, IgG1 and IgG4 subclasses whilst inhibiting IgG3 production (Lundgren et al., 1989; Pene et al., 1988b). In contrast, IgE and IgG1 antibody production is inhibited by IFN-γ (Pene et al., 1988a). In addition, IgA antibody is produced in the presence of TGF-β (Cerutti, 2008). A summary of the role cytokines play in inhibiting or stimulating class switching in B cells is shown in ***Table 1.2***.

The co-stimulatory receptor CD40 and its ligand (CD40L) also play a pivotal role in class switching and B cell activation (Fuleihan et al., 1993; Wykes, 2003). Those lacking functional CD40L on their B cells suffer from a disease known as hyper-IgM syndrome characterised by normal to high levels of circulating IgM and very low concentrations of all other classes of antibody (Aruffo et al., 1993).

Although able to produce antibodies of all classes, antibody responses to TD antigens are predominantly IgG1 and responses TI antigens are generally restricted to IgM, IgG2 and IgG1 (Barrett and Ayoub, 1986; Stevens et al., 1983). TD antigens are strongly immunogenic producing high-affinity antibody and immunological memory whereas TI antigens are poorly immunogenic and produce relatively low-affinity antibodies and no immunological memory (Richmond et al., 2000). Meningococcal polysaccharides are TI-2 antigens and are poorly immunogenic inducing primarily IgM, IgG2 and IgG1 antibody (de Voer et al., 2011). Low-affinity antibodies are associated with lower SBA titres (Hetherington and Lepow, 1992). Additionally, IgG2 antibodies are poor activators of complement, an important mechanism in the protection

against meningococcal disease (Augener et al., 1971). As such, meningococcal polysaccharides are conjugated to proteins to drive the antibody response towards that exhibited by TD antigens (Pace et al., 2009). Indeed, meningococcal protein conjugate vaccines produce higher SBA titres and induce some immunological memory (Galson et al., 2015; Memish et al., 2011; Shao et al., 2009).

1.2.5 Complement Activation by Immunoglobulins

As previously discussed, IgG and IgM antibodies activate the complement system when bound to antigen. Both antibody subclasses activate complement by binding the complement protein C1q. The affinity of C1q for immunoglobulin and antigen density is thought to dictate the level of complement activation.

1.2.5.1 Human C1q

First assigned the nomenclature of 11S due to its sedimentation velocity, C1q was discovered based on its ability to bind aggregated IgG antibody and its role in complement activation (Müller-Eberhard and Kunkel, 1961). The 11S protein was found in complex with two proenzymes, later termed C1r and C1s (Lepow et al., 1963). Early studies on the structure of C1q showed it to be composed of two non-covalently associated subunits with molecular weights of 60kDa (C1qI) and 42kDa (C1qII), respectively (Yonemasu and Stroud, 1972). C1qI was revealed as a disulphide-bonded, heterodimer formed of two subcomponents of 29kDa (C1qI-1) and 27kDa (C1qI-2). C1qII was revealed as a disulphide-bonded, homodimer formed of a 22kDa subcomponent (Reid, 1976; Yonemasu and Stroud, 1972). The three subcomponents of C1q are present in equal concentrations and have subsequently been assigned the nomenclature of a-chain, b-chain and c-chain of C1q (Reid et al., 1972). Electron microscopy experiments with negatively stained C1q revealed it to consists of six terminal subunits each joined to a central

core by connecting strands (Knobel et al., 1975; Shelton et al., 1972). Amino acid composition studies showed the abundance of hydroxyproline and hydroxylysine, typically found in collagen and not globular proteins (Reid et al., 1972). Inactivation of C1q by incubation with collagenase enzyme further pointed to a collagen-like region of the protein. Amino acid sequencing confirmed that each of the chains contains an N-terminal collagen-like region and C-terminal non-collagen-like or globular region (Reid, 1974). The collagenous region of each subunit (composed of an a-chain and b-chain dimer and one-half of a c-c-chain dimer) form a helical triple helix with each helix held together by the covalent bond between each c-chain dimers (Reid and Porter, 1976). The structure and organisation of C1q are shown in ***Figure 1. 5***.

1.2.5.2 C1q Affinity to Immunoglobulins

The binding affinity of monomeric IgG to C1q is weak, reported to range between 1.1 and 23.9 x 10^{-4}M for the different human IgG subclasses (Hughes-Jones, 1977; Hughes-Jones and Gardner, 1979; Painter et al., 1982; Schumaker et al., 1976; Sledge and Bing, 1973). The affinity of C1q is in the order IgG3>IgG1>IgG2>>IgG4 (Schumaker et al., 1976). The binding affinity is significantly increased upon aggregation of IgG by heat treatment or clustering when bound to antigen. Binding of C1q to multiple IgG ligands increases the avidity by several logs of magnitude into the nanomolar scale (Bindon et al., 1988; Emanuel et al., 1982; Hughes-Jones, 1977; Moore et al., 2010; Painter et al., 1982; Patel et al., 2015; Quast et al., 2015). The binding affinity of fluid-phase IgM antibody to human C1q is in the micromolar range despite possessing five times the number of binding sites of IgG (Poon et al., 1985; Sledge and Bing, 1973); however, binding affinity markedly increases following interaction with antigen due to a conformational change of IgM from its planar to staple form (Burton, 1986; Swanson et al., 1988; Wright et al., 1990; Wright et al., 1988).

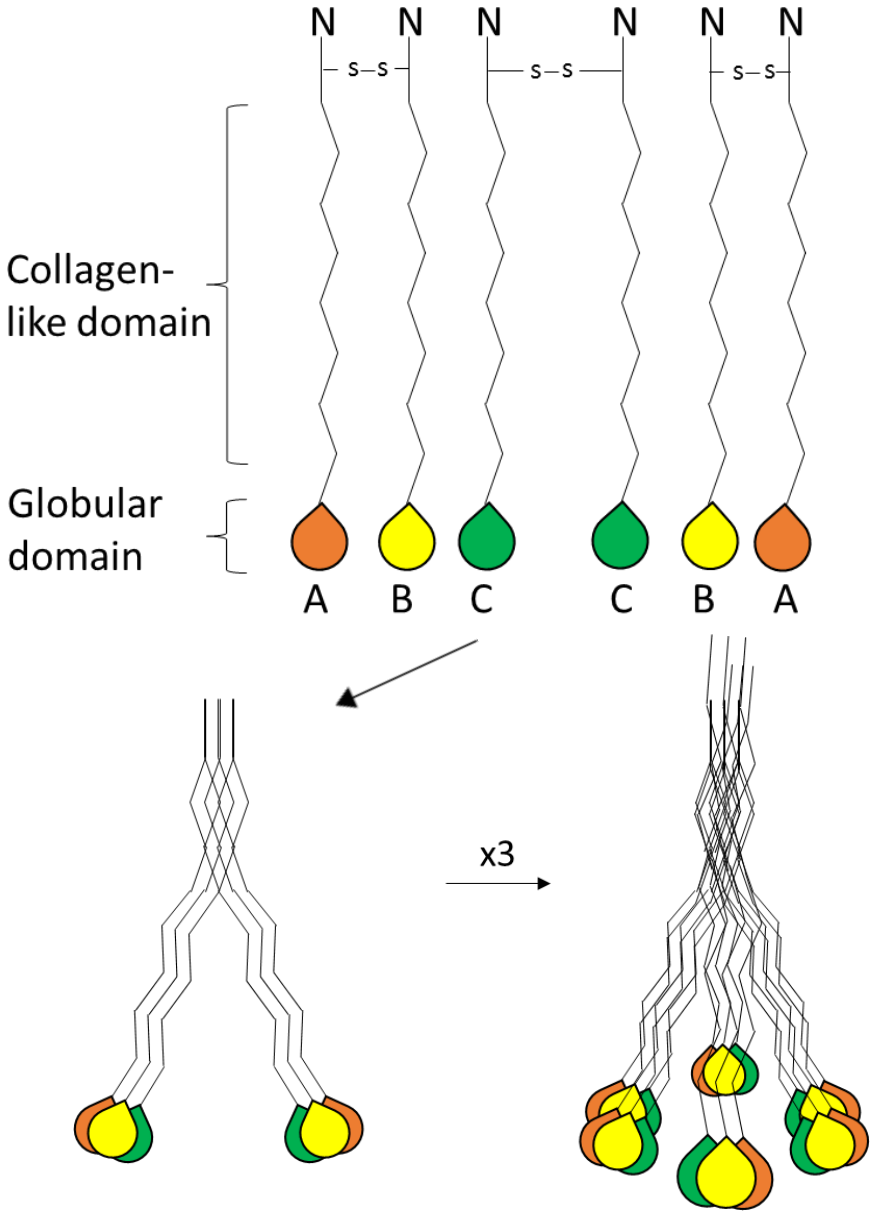


Figure 1. 5 Structure of C1q

C1q is composed of 6 x 3 polypeptide chains (A, B and C chains) held together by covalent and non-covalent interactions. Each chain is formed of a short non-collagenous region at the N-terminus of the protein followed by a longer collagen-like domain and globular domain. Chains A-B and C-C are covalently linked by a disulphide bond at the non-collagenous region at the N-terminus. The collagenous region of each subunit (composed of an A-chain and B-chain dimer and one-half of a C-dimer) form a helical triple helix with each helix held together by the covalent bond between each C-chain dimers.

Contemporary studies of the binding affinity of C1q to immunoglobulins have been achieved with surface plasmon resonance (SPR) (Moore et al., 2010; Patel et al., 2015; Quast et al., 2015). Immobilisation of antibody for C1q binding studies has been conducted using three different methods: direct coupling, captured with Protein A or captured with Protein L. The C1q-binding affinity (KD) of a human IgG1 antibody directly amine coupled to an SPR sensor chip was calculated as 3.5nM (Quast et al., 2015). The KD of a human IgG1 antibody captured by a Protein A coated SPR sensor chip was calculated as 48nM (Moore et al., 2010). Both these methods have been criticised as the alignment of the antibody is not optimal for interaction with C1q (Patel et al., 2015). Protein L binds to the light chain of immunoglobulins and thereby orientates antibodies for efficient interaction with C1q (Graille et al., 2001). Using a Protein L coated SPR sensor chip to capture antibody, the KD of human C1q to human IgG1, IgG2, IgG3 and IgG4 was calculated as 81nM, 191nM, 88nM and 223nM respectively (Patel et al., 2015).

The flexibility of IgG antibodies is determined by the length of the hinge connecting CH1 and CH2 domains (Feinstein, 1965; Valentine and Green, 1967). Specifically, the length of the upper hinge region is proportional to the degree of flexibility offered between the Fc and Fab regions of an antibody (Dangl et al., 1988; Oi et al., 1984). It is thought that the hinge length of an antibody also correlates with its ability to fix complement. For humans, IgG3 is the most flexible, followed by IgG1, IgG2 and IgG4. It is hypothesised that increased flexibility allows efficient organisation of multiple Fc regions to interact with C1q, a necessity for activation. A longer hinge also moves the Fab regions of an antibody far enough away from the C1q binding motif not to restrict binding. Indeed, immunoglobulins with their hinge region deleted are rendered inflexible and incapable of complement activation (Deutsch and Suzuki, 1971; Klein et al., 1981). Inflexibility and steric hindrance of the C1q binding motif by the Fab region were proposed as one of the main reasons for the inability of human IgG4 antibody to activate

complement. This was further strengthened when the Fc fragment of IgG4 was shown to bind C1q to a similar extent to the Fc fragment of IgG1 (Isenman et al., 1975).

Additional investigations have shown that differences in hinge length and antibody flexibility do not fully account for variations in complement activation (Brekke et al., 1995; Michaelsen et al., 1994; Norderhaug et al., 1991; Tan et al., 1990). Chimeric human IgG3 and IgG4 antibodies, each with their hinge region replaced with the other, showed no significant difference in C1q binding compared to their native counterparts (Tan et al., 1990). For example, an IgG4 antibody with the hinge of IgG3 did not activate complement and vice versa. However, total deletion of the hinge region again removed the capability of IgG3 to activate complement. Further scrutiny of the hinge region of IgG3 showed that the hinge region was not necessary to bind C1q and fix complement (Brekke et al., 1995; Michaelsen et al., 1994). However, a disulphide bond, generated by mutation of the N-terminus of the CH2 domain correctly aligning the two CH2 domains of the antibody, was required for complement activation.

A recent study, investigating the importance of antibody clustering and antigen density in classical pathway activation, described the ability of IgG1 antibodies to form hexamers by interacting through their Fc domains (Diebolder et al., 2014). Mutations in the CH2 and CH3 domains, enhancing the interaction of multiple antibodies (Glu^{345} to Arg), increased complement activation for IgG1, IgG2, IgG3 and IgG4 to similar levels. It is hypothesised that antigen density, hinge length and flexibility play a role in hexamer formation. This phenomenon of the polymerisation of immunoglobulins at sites of high antigen density was previously predicted when comparing the differences in the ability of IgM and IgG antibody to activate complement (Burton, 1986; Watts et al., 1985).

1.2.5.3 C1q Binding Motif on Human Immunoglobulins

The antibody fragment Fabc, containing the VH1, CH1 and CH2 domains, retains the ability to activate complement (Colomb and Porter, 1975). This, with the knowledge that Fab fragments (containing VH1-CH1 domains) do not fix complement, and that Fc fragments (containing CH2-CH3 domains) suggest that the C1q binding motif must be contained within the CH2 domain of IgG. This was further confirmed when the functional affinity of IgG sub-fragments to C1q was assessed (Painter et al., 1982). The functional affinity of the Fc fragment and CH2 sub-fragment were both measured at 4 x $10^4 M^{-1}$. In contrast, the IgG sub-fragment CH3 expressed no affinity to C1q.

Mutational and sequencing studies of the CH2 domain have further scrutinised the C1q binding motifs on IgG. The first mutational study, investigating the C1q binding site on immunoglobulins, was performed with a mouse IgG2b antibody (Duncan and Winter, 1988). The amino acid residues Asparagine^{297}, Glutamic acid^{318}, Lysine^{320} and Lysine^{322} in the CH2 domain of mouse IgG2b were identified as essential for interaction with C1q. These residues are conserved in the immunoglobulins of multiple species, including all human IgG subclasses, and this region was predicted to be the C1q binding motif for all complement fixing immunoglobulins. Additional investigations, carried out to improve the understanding of the differences between human IgG subclasses and their interaction with C1q, have painted a more complex picture (Gaboriaud et al., 2003; Idusogie et al., 2000; Moore et al., 2010; Morgan et al., 1995; Schneider and Zacharias, 2012; Thommesen et al., 2000; Xu et al., 1994).

Several reports investigating the binding site of C1q for IgG1 antibody showed that the previously predicted residues Lysine^{320} and Glutamic acid^{318} are not essential for interaction with C1q (Idusogie et al., 2000; Morgan et al., 1995; Thommesen et al., 2000). However, residues Aspartic acid^{270}, Lysine^{322}, Proline^{329}, and Proline^{331} were all implicated. Not only have residues been identified that, when mutated, reduced C1q binding, but also other mutations described

that can enhance the interaction (Moore et al., 2010). A total of twelve residues within the CH2 domain of IgG1 are predicted to interact with C1q (Schneider and Zacharias, 2012). These include the residues Aspartic acid^{270}, Lysine^{322}, Proline^{329} and Proline^{331} also shown to be important experimentally. The highly conserved glycosylation site at Asparagine^{297} also plays an important role in C1q binding (Nose and Wigzell, 1983; Quast et al., 2015). De-glycosylation or removal of this site significantly reduces C1q binding. ***Table 1. 3*** shows the full sequence of the CH2 domains of the human IgG subclasses, residues identified as important for C1q binding and areas of variability between the subclasses.

Whilst human IgG4 antibody shares many residues with IgG1 highlighted as essential for binding, including those originally identified on mouse IgG2b, it interacts poorly with C1q. The amino acid residue Serine^{331}, which only occurs in IgG4, is thought to be partly responsible for this lack of affinity (Brekke et al., 1994; Tao et al., 1993; Xu et al., 1994). Replacing Proline^{331} in IgG1 and IgG3 antibodies for Serine abolishes C1q binding in these subclasses whilst a Serine^{331} to Proline substitution in IgG4 can recover binding (Xu et al., 1994).

1.2.5.4 Immunoglobulin Binding Motif on C1q

C1q binds antibody through the globular heads at the C-terminus of the molecule, denoted by size as A, B and C (Duncan and Winter, 1988; Hughes-Jones and Gardner, 1979). When individually isolated, each of the globular heads show binding to IgG and IgM (Kaul and Loos, 1997). A more detailed analysis of individual globular head binding to antibody showed preferential binding of IgG to globular heads A and B over C whilst IgM showed preferential binding to globular heads A and C (Kishore et al., 2003; Kishore et al., 1998).

As with the binding motif for C1q on IgG, mutations of the residues predicted as potential points of interaction have been performed with each globular head of C1q (Gadjeva et

al., 2008; Kojouharova et al., 2004; Roumenina et al., 2006; Zlatarova et al., 2006). These studies have revealed a bias in the contribution that globular head B makes in antibody binding. For IgG1, the residues Arginine^{A162}, Lysine^{A200}, Arginine^{B108}, Arginine^{B109}, Arginine^{B114}, Histidine^{B117}, Arginine^{B129}, Lysine^{B136}, Arginine^{B163} and Arginine^{C156} have all been implicated. A total of twenty residues from globular heads B and C are predicted to facilitate the binding to IgG (Gaboriaud et al., 2003; Schneider and Zacharias, 2012). The binding motif for IgM is less well studied. The amino acid residues Tyrosine^{B175}, Arginine^{B108}, Arginine^{B109}, Lysine^{B136}, Lysine^{A200}, Histidine^{C101} and Lysine^{C170} have each been indicated in IgM binding (Gadjeva et al., 2008; Zlatarova et al., 2006). These residues, along with those highlighted for IgG, are detailed in ***Table 1. 4***.

Table 1. 3 C1q Binding Motifs on Rabbit IgG and Human IgG Subclasses

The amino acid sequences of the hinge and CH2 regions of rabbit IgG and human IgG subclasses is shown. Amino acid residues contained within the CH2 regions identified to reduce, facilitate or are not involved in the interaction with C1q are highlighted. The glycosylation site and residues that vary between antibodies are also highlighted.

Region	Amino Acid Sequence
IgG1 Hinge	EPKSCDKTHTCP
IgG2 Hinge	ERKCCVECPPCP
IgG3 Hinge	ELKTPLGDTTHTCPRCPEPKSCDTPPPCPRCPEPKSCDTPPPCPRCPEPKSCDTPPPCPRCP
IgG4 Hinge	ESKYGPPCPSCP
Rabbit Hinge	SKPTCPPPEL
IgG1 CH2	PCPAPEL^{2}L^{2}GGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS^{1}H^{1}ED^{2}PEVK^{3}FNWYVDGVEVHNAKTKPREEQYN^{297}STYRVVSVLTVLHQDWLNGK*E*^{3}Y^{2}*K*^{3}CKVS^{1}NK^{1}ALP^{2}AP^{2}IE^{1}KTISKAK
IgG2 CH2	APPVA^{1}GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQFNWYVDGVEVHNAKTKPREEQFN^{297}STFRVVSVLTVVHQDWLNGKEK^{2}*KCK*VSNKGLPAP^{2}IEKTISKTK
IgG3 CH2	APEL^{2}L^{2}GGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHED^{2}PEVQFK^{2}WY^{3}VD^{3}GVEVHNAKTKPREEQYN^{297}STFRVVSVLTVLHQDWLNGKEY^{2}KC*K*^{2}VSNKALP^{2}AP^{2}IEKTISKTK
IgG4 CH2	APEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFN^{297}STYRVVSVLTVLHQDWLNGKEY^{2}KCKVSNKGLPSS^{1}IEKTISKAK
Rabbit CH2	LGGPSVFIFPPKPKDTLMISRTPEVTCVVVDVSQDDPEVQFTWYINNEQVRTARPPLREQQFN^{297}STIRVVSTLPITHQDWLRGKEY^{2}KC*K*^{2}VHNKALPAP^{2}IEKTISKARGQPLEPK

Table Key: *A*^{1} = Reduce interaction with C1q; N^{297} = Glycosylation site; D^{2} = Enhance interaction with C1q; D^{3} = Not involved in C1q binding; **D** = Areas of variation.
Table References: (Brekke et al., 1994; Duncan and Winter, 1988; Gaboriaud et al., 2003; Idusogie et al., 2000; Moore et al., 2010; Morgan et al., 1995; Nose and Wigzell, 1983; Quast et al., 2015; Schneider and Zacharias, 2012; Tao et al., 1993; Thommesen et al., 2000; Xu et al., 1994).

Table 1. 4 IgG and IgM Binding Motifs on C1q

The amino acid sequences of the A, B and C chains of human C1q. Residues indicated in binding IgG or IgM, IgG and IgM are highlighted. Residues underlined and emboldened are contained within the globular heads of each chain.

C1q component	Sequence
A chain	MEGPRGWLVLCVLAISLASMVTEDLCRAPDGKKGEAGRPGRRGRPGLKGEQGEPGAPGIRTGIQGLKGDQGEPGPSGNPGKVGYPG PSGPLGARGIPGIKGTKGSPGNI**KDQPRPAFSAIRRNPPMGGNVVIFDTVITNQEEPYQNHSGRFVCTVPGYYYFTFQVLSQWEICLSI VSSSRGQVR_2RSLGFCDTTNKGLFQVVSGGMVLQLQQGDQVWVEKDPKK^+GHIYQGSEADSVFSGFLIFPSA**
B chain	MMMKIPWGSIPVLMLLLLLGLIDISQAQLSCTGPPAIPGIPGIPGTPGPDGQPGTPGIKGEKGLPGLAGDHGEFGEKGDPGIPGNPGKV GPKGPMGPKGGPGAPGAPGPKGESGDY**KATQKIAFSAT_2RT_2INVPLR^+R^+DQTIR_2FDH_2VI_2TNMNNNYEPR_2SGK_2FTC K^+VPGLYYFTYHASSRGNLCVNLM_2RG$R_2$$E_2$$R_2$AQKVVTFCDYA$Y_1$NTFQVTTGGMVLKLEQGENV$F_2$LQATDKNSLLGME GANSIFSGFLLFPDMEA**
C chain	MDVGPSSLPHLGLKLLLLLLLLPLRGQANTGCYGIPGMPGLPGAPGKDGYDGLPGPKGEPGIPAIPGIRGPKGQKGEPGLPGHPGKNGP MGPPGMPGVPGPMGIPGEPGEEGRY**KQKFQSVFTVTRQTH_1QPPAPNSLIRFNAVLTNPQGDYDTSTGKFTCKVPGLYYFVYHAS HTANLCVLLYR_2SGV_2KV_2VTFCGHTSK_1TNQVNSGGV_2LLRLQVGE_2EVWLAVNDYYDMVGIQGSDSVFSGFLLFPD**

Table Key: M_2 = IgG binding only; Y_1 = IgM binding only; R^+ = IgG and IgM binding. **Table References**: (Gaboriaud et al., 2003; Gadjeva et al., 2008; Kishore et al., 2003; Kishore et al., 1998; Kojouharova et al., 2004; Roumenina et al., 2006; Schneider and Zacharias, 2012; Zlatarova et al., 2006).

1.2.5.5 Complement Activation and Antigen Density

Of the human antibody subclasses, IgM, IgG3 and IgG1 exhibit the greatest ability to activate complement (Bindon et al., 1988; Brüggemann et al., 1987; Dangl et al., 1988; Garred et al., 1989). The IgG subclasses IgG2 and IgG4 show very little complement activation (Davies and Sutton, 2015; Xu et al., 1994). In lower antigen densities, IgG3 shows better complement activation than IgG1 whilst at higher antigen densities it is IgG1 that shows better complement activation that IgG3 (Garred et al., 1989; Giuntini et al., 2016).

It was thought that this apparent resilience of IgG3 to fix complement, even in areas of low antigen density, may be explained by its extended hinge region and resultant increased flexibility compared to the other human IgG subclasses (Edelman et al., 1969; Michaelsen et al., 1977; Vidarsson et al., 2014). However, this hypothesis was recently challenged when the ability of IgG1, IgG3 and hinge-truncated IgG3 antibodies to fix C1q and activate complement on the surface of *Neisseria meningitidis* bacteria was assessed (Giuntini et al., 2016). As before, IgG3 activated complement better that IgG1 when bound to a sparsely expressed antigen and IgG1 activates better than IgG3 when bound to a densely expressed antigen. Unexpectedly, the IgG3 hinge-truncated mutants had enhanced complement activation when compared to intact IgG3 suggesting the long IgG3 hinge is not responsible for the superior complement activation compared to IgG1 with sparse antigens. These observations confirm previous findings that a hinge-truncated IgG3 antibody had superior complement activation capabilities compared to wild type IgG3 (Norderhaug et al., 1991). The exact mechanism behind this phenomenon is unknown although it is suspected that the long hinge of IgG3 keeps complement activation further from the antigenic surface than IgG1 reducing potency.

1.3 The Complement System

The complement cascade is an ancient part of the innate immune system comprised of a series protein components found in the blood (Dunkelberger and Song, 2009). The complement system functions to clear pathogens from the host by several mechanisms including opsonisation, complement dependent cytotoxicity and chemotaxis (Frank and Fries, 1991; Nuttall, 1888; Ward et al., 1965). Three pathways of complement activation, known as the classical, lectin and alternative pathways, have been described (Arlaud and Colomb, 2005; Thurman and Holers, 2006; Turner, 1996). Each of these pathways share many components but are activated by distinct mechanisms. An overview of complement activation for each pathway is shown in ***Figure 1. 6***.

The discovery of complement has been attributed to a number of scientists in the late 19th and early 20th century who noted the bactericidal effects of serum. Élie Metchnikoff (1845-1916), Russian zoologist and former pupil of Pasteur, was the first to describe the ability of phagocytic cells to clear infection by engulfing and digesting them (Gordon, 2008). At the time it was thought that phagocytosis was the main action of pathogen clearance until Hungarian scientist Josef Von Fordor (1843-1901), working on Anthrax, showed that the acellular fraction of blood alone could directly kill the bacteria (Tauber and Chernyak, 1989; Von Fodor, 1886). German bacteriologist Hans Buchner (1850-1902) who also noticed this phenomenon coined the term 'alexin' from the Greek 'to ward off' (Buchner, 1891; Buchner, 1889). Bacteriologists Richard Pfeiffer (1858-1945), George Nuttall (1862-1937) and Jules Bordet (1870-1961) showed the lytic properties of blood were enhanced with prior vaccination but inhibited when heated to 56^{o}C suggesting that alexin was, in fact, two separate entities (Nuttall, 1888; Pfeiffer, 1894). Bordet proposed that bacteria were first sensitised by binding antibodies allowing the complement system to deliver the killer blow (Bordet, 1895; Bordet, 1900). Building on this work Professor Paul Ehrlich (1854-1915) noted in his 1899 work entitled 'Zer Theorie der

Lysinwirkung', or 'On the theory of the action of lycin', that the heat liable component of alexin *complemented* the action of antibodies in the destruction of bacteria (Ehrlich and Morgenroth, 1899). As such, the term 'complement' is now used to describe the heat-labile component responsible for the bactericidal activity of blood. The idea of complement existing of multiple components was first demonstrated in 1907 by A. Ferrata and E. Brand (Brand, 1907; Ferrata, 1907). Today, more than 30 components of complement have been described, and its importance in disease is well understood (Sarma and Ward, 2011).

1.3.1 Pathways of Complement Activation

1.3.1.1 Classical and Lectin Pathways

The classical pathway of complement was the first pathway of activation to be described following the experiments of Bordet, Ehrlich and Morgenroth (Bordet, 1895; Bordet, 1898; Ehrlich and Morgenroth, 1899; Ehrlich and Morgenroth, 1901). Whilst investigating the lytic properties of blood, they noted that complement dependent cytotoxicity of erythrocytes was only initiated once first sensitised by antibody. The classical pathway is triggered by binding of the C1 complex to its ligand (Cooper, 1985). The C1 complex is composed of a recognition domain, C1q, and a serine protease domain, comprising of two copies each of C1r and C1s (Lepow et al., 1963). The C1r and C1s tetramer interact with each of the six triple helical collagen-like stems protruding from each globular head of C1q which is stabilised in the presence of Ca^{++} ions (Gregory et al., 2003). The immunoglobulins IgG and IgM are the most studied ligands of C1q, but many other ligands have been described (Gaboriaud et al., 2003).

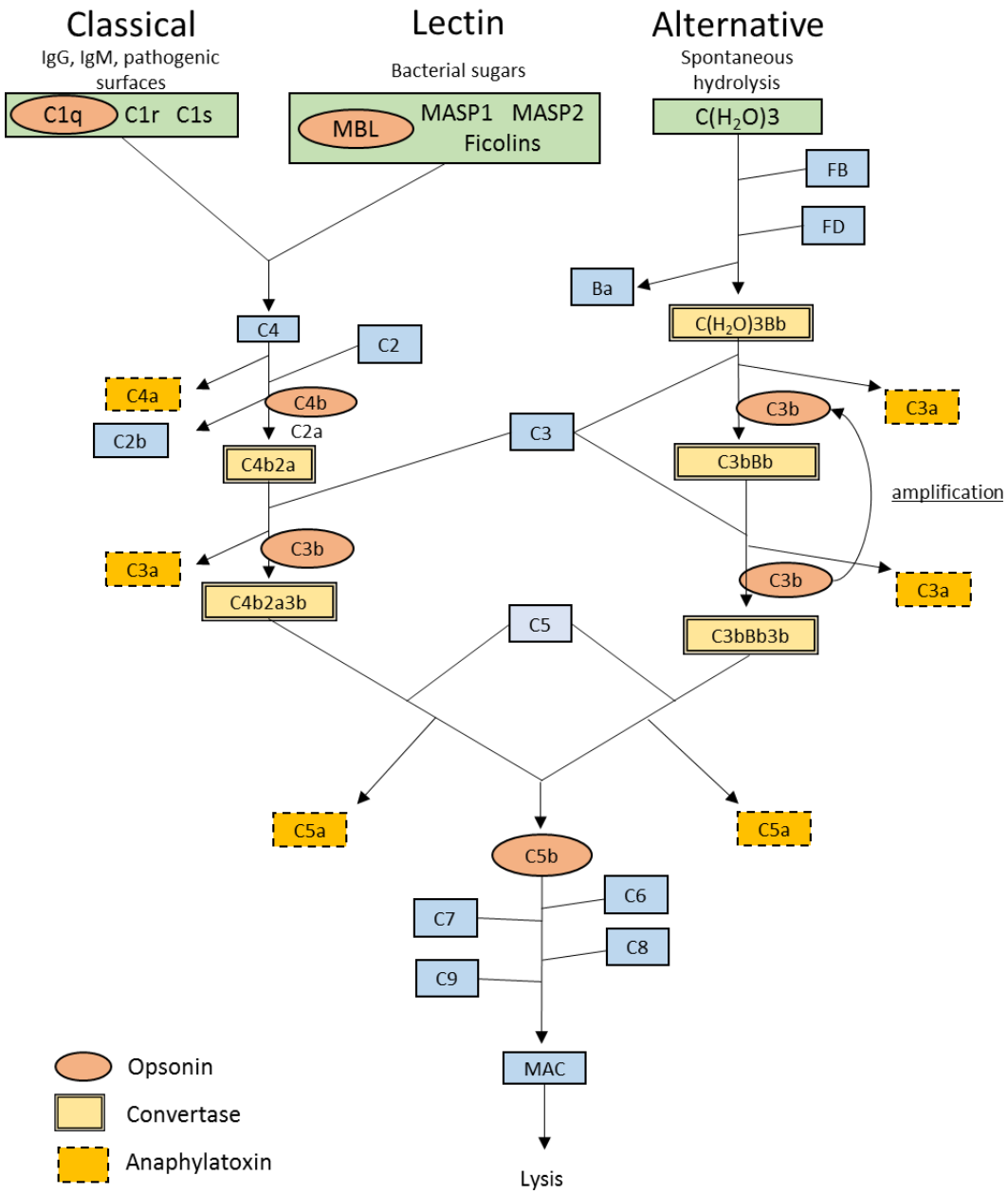


Figure 1. 6 Pathways of Complement Activation

The complement system is activated by three different pathways: classical, lectin and alternative. The classical and lectin pathways are activated by the serine protease associated pattern recognition molecules C1q and MBL/ficolins, respectively. The conformational changes in C1q and MBL that occur when bound to their respective ligands activate the associated serine proteases, which in turn cleave C4 and C4b-bound C2 forming the C3 convertase of the classical and lectin pathways, C4b2a. The alternative pathway is activated by spontaneous hydrolysis of C3 causing a conformational change that allows FB to associate. FD then cleaves FB to generate the C3 convertase of the alternative pathway, $C3(H_2O)3Bb$. The C3 convertases of each pathway cleave C3 into C3a and C3b. The associated of C3b with each C3 convertases forms the C5 convertase for each three pathways. The C5 convertase cleaves C5 into C5a and C5b. C5b further associated with C6, C7, C8 and C9 to form the membrane attack complex (MAC). Proteolytic fragments C4a, C3a and C5a function as anaphylatoxins whereas the fragments C4b, C3b and C5b function as opsonins.

The lectin pathway of complement activation is the most recent of the three pathways to be described following the isolation of mannose-binding lectin (MBL), the activator of the lectin pathway, in the late 1970s and 1980s (Ikeda et al., 1987; Kawasaki et al., 1983; Kawasaki et al., 1978). Noting the similarities between C1q and MBL, Y. Kozutsumi, T. Kawasaki and I. Yamashina postulated its ability to activate complement when bound to cell surfaces (Ikeda et al., 1987). Unlike the classical pathway where C1q is the only recognition protein, the lectin pathway consists of two distinct families of recognition proteins: ficolins and collectins (Holmskov et al., 2003). MBL is a member of the collectin family and the best characterised of the lectin pathway activators. Like C1q, MBL associates with a tetramer of serine proteases, two monomers each of MBL-associated serine protease-1 and 3 (MASP-1 and 3) and MASP-2 (Dahl et al., 2001; Matsushita and Fujita, 1992; Thiel et al., 1997). MBL monomers are formed of a short non-collagenous region at the N-terminus of the protein followed by a longer collagen-like domain, a neck region and carbohydrate recognition domain (Dong et al., 2007). Homotrimers, known as stalks, associate through the interaction of collagen-like regions between each monomer stabilised by disulphide bonds between the N-terminus regions (Thiel, 2007). Finally, two to six stalks assemble forming functional MBL. Only one dimer of MASP-1 or MASP-2 tends to associate with the collagen-like domain of MBL trimers and tetramers whereas MASP-2 and MASP-3 tend to associate with the collagen-like domain of higher oligomers of MBL (Dahl et al., 2001). In serum, MBL predominantly exists as a trimers or tetramers. MBL binds 3- and 4-hydroxyl groups in the pyranose ring carbohydrates, such as glucose, *D*-mannose and *N*-acetylglucosamine, commonly expressed by pathogenic cells, in the presence of Ca^{++} ions (Endo et al., 2006).

Multivalent binding of the C1 complex causes a conformational change bringing the activation and catalytic sites of both C1r proteases together (Gaboriaud et al., 2004). C1r is then auto-activated by the cleavage of the arginine–isoleucine bond in the activation site of one C1r monomer by a serine residue within the catalytic site of the other (Lacroix et al., 2001). Once activated, C1r then cleaves and activates C1s in the same fashion. Activated C1s shows specificity for both C4 and C2. C1s first cleaves C4, into C4a and C4b, and then cleaves C2, into C2a and C2b, but only when C2 is in complex with C4b (Müller-Eberhard et al., 1967; Polley and Müller-Eberhard, 1968). Unlike C1r, MASP-1 dimers are not auto-activated but activated by a separate MASP-1 dimer when MBL complexes cluster (Kjaer et al., 2016; Kjaer et al., 2015). Activated MASP-1- cleaves and actives MASP-2 which can then cleaves C4 and C4b-bound C2 (Héja et al., 2012). MASP-1 also shows serine protease activity towards C4-bound C2 (Møller-Kristensen et al., 2007). The larger fragments of proteolysis, C4b and C2a, remain together forming the C3 convertase of the classical and lectin pathways, C4bC2a, whereas C4a and C2b are released. C4b, anchored to the surface of pathogens, is an opsonin targeting cells for phagocytosis (Krych-Goldberg and Atkinson, 2001). Upon the cleavage of C4, an internal thioester is exposed covalently linking C4b to amino or hydroxyl groups present on pathogenic surfaces (Dodds et al., 1996). This same mechanism also tethers C3b to pathogenic surfaces (Sim et al., 1981).

The preferred substrate of C4b2a is C3 (Pangburn and Rawal, 2002). However, C5 may also associate with C4b2a but with a 1000-fold lower affinity than C3 (Rawal and Pangburn, 2003). Cleavage of C3 into C3b and C3a is executed by the serine protease domain of C4b-bound C2a (Krishnan et al., 2007). The specificity of the convertase dramatically alters to C5 upon the binding of C3b to the alpha chain of C4b through an ester bond (Takata et al., 1987). The C3a fragment is released into the circulation and C3b that does not associate with C4b2a may bind directly to the surface of the pathogen via the same exposed thioester, opsonising those cells for phagocytosis (Fearon, 1980; Reid and Porter, 1981). C3a is an anaphylatoxin able to

chemotactically attract leukocytes to areas of complement activation (Ward et al., 1965). C5 can then be cleaved by the C5 convertase (C4b2a3b) generating C5a and C5b. C5a, another anaphylatoxin, is released into the circulation (Snyderman et al., 1970). C5b associates with C6, C7, C8 and C9 forming the membrane attack complex (MAC) (Serna et al., 2016).

1.3.1.2 Alternative Pathway

The alternative pathway of complement activation was first proposed in the 1950s by Louis Pillemer et al., following the discovery of properdin (from the Latin *perdere* meaning 'to destroy'), now known to be positive regulator of the alternative pathway (Nesargikar et al., 2012; Pillimer et al., 1954).

The alternative pathway becomes activated by the spontaneous hydrolysis of the internal thioester bond of C3 into C3(H_2O), a C3b-like molecule, at a rate of roughly 0.005% per minute (Pangburn et al., 1981). In addition, hydrolysis of C3 into C3 (H_2O) also reveals the factor B (FB) binding region, allowing the formation of the pro-convertase complex C3(H_2O)B in the presence of Mg^{++} (Forneris et al., 2010). The association of FB with hydrolysed C3 (or C3b) exposes the internal scissile bond in FB for cleavage by the serine protease factor D (FD) into Bb and Ba (Milder et al., 2007). In solution, FD is self-inhibited by a structural loop masking the active site that is only revealed upon interaction with C3 (H_2O) -bound (or C3b-bound) FB (Jing et al., 1998). Ba is released into the circulation whereas Bb remains bound to C3b generating the C3 convertase of the alternative pathway, C3bBb (Rooijakkers et al., 2009). Properdin binding to both C3b and Bb stabilises this convertase by slowing disassociation and inhibiting cleavage by factor I (FI) in the presence of FH promoting complement activation (Alcorlo et al., 2013; Fearon and Austen, 1975; Hourcade, 2006).

The substrate for C3bBb is C3, cleaving the scissile bond releasing C3a into the circulation leaving C3b to bind and opsonise cell surfaces and complex with C3bBb (Reid and Porter, 1981). Membrane-bound C3b can again associate with FB forming more C3 convertase complexes, cleaving even more C3 driving complement activation in a process known as the amplification loop of the alternative pathway (Lachmann, 2009; Müller-Eberhard and Götze, 1972). The amplification loop of the alternative pathway also amplifies the classical and lectin pathways once they themselves are activated (Harboe et al., 2004). Alternative pathway inhibition assays show that roughly 80 to 90% of complement activation by the classical pathway can be attributed to alternative pathway amplification. The association of additional membrane-bound C3b molecules with the C3bBb complex forms the C5 convertase of the alternative pathway, C3bBb3b (Berends et al., 2015; Daha et al., 1976; Kinoshita et al., 1988). C5 is then cleaved by the C5 convertase generating C5a, which is released into the circulation, and C5b, which associates with C6, C7, C8 and C9 forming the MAC (Serna et al., 2016).

1.3.2 Functions of Complement

1.3.2.1 Complement Dependent Cytotoxicity

Pathogens are directly lysed by complement in a process known as complement mediated cytotoxicity (CDC) (Nuttall, 1888). Regardless of the pathway of complement activation initially triggered, sufficient complement activation results in the formation of pores in the lipid bilayer of cellular membranes (Muller-Eberhard, 1988). Known as the membrane attack complex (MAC), these pores are composed of the complement components C5b, C6, C7, C8 and C9 in a molar ratio of 1:1:1:1:19-22, respectively (Dudkina et al., 2016; Serna et al., 2016). CDC is of particular importance in the clearance of Gram-negative bacteria, such as *Neisseria*

meningitidis (Taylor, 1983). Individuals deficient in the components of MAC are vulnerable to recurrent meningococcal disease (Ross and Densen, 1984)

Insertion of MAC through the lipid bilayer of pathogens disturbs the permeability and stability of the membrane disrupting the osmotic gradient between intercellular and extracellular ions (Esser, 1994). The rapid flux of extracellular fluid into the cell and extrusion of intracellular material leads to cell death (Podack, 1986). MAC attack that does not directly result in cell death can initiate downstream pathways involved in proliferation, apoptosis, transcription, lipid metabolism and inflammation (Morgan, 2016; Morgan and Campbell, 1985; Takano et al., 2013; Triantafilou et al., 2013).

The first event in MAC formation is the cleavage of C5 by the C5 convertase of either classical/lectin (C4b2a3b) or alternative (C3bBbC3b) pathways into C5a and C5b tethered to the surface of the targeted cell (Medicus et al., 1976; Pangburn and Rawal, 2002). C5b forms an irreversible complex with C6 and C7 in the fluid-phase followed by C8 and C9 once membrane-bound (Aleshin et al., 2012). Binding of C7 to the C5b6 complex causes a significant conformational change exposing the hydrophobic stalk of C7 thus anchoring C5b67 into but not through the lipid bilayer (DiScipio et al., 1988). C8 is formed of three chains: α, β and γ. Membrane-bound C5b67 binds to the β chain of C8 causing a conformational change with the α and β chains of C8 inserting their hydrophobic regions into the membrane further anchoring the complex (Tegla et al., 2011). C9 then binds the α chains of C8 unravelling C9 through the lipid membrane and allowing the addition of further C9 molecules.

1.3.2.2 Inflammation

Complement recruits leukocytes to areas of complement activation by the process of chemotaxis (Ward et al., 1965). Complement chemoattractants, also known as anaphylatoxins, include C5a and C3a (Frank and Fries, 1991). Anaphylatoxins were named as such due to their ability to induce cardiac dysfunction and hypersensitivity reactions in guinea pigs (Del Balzo et al., 1985). The complement component C1q, acting through C1q receptors expressed by neutrophils, mast cells, DCs and eosinophils, is also a chemoattractant (Eggleton et al., 1998; Ghebrehiwet et al., 1995; Kuna et al., 1996; Vegh et al., 2006). Generated in large quantities at sites of complement activation, anaphylatoxins diffuse into the circulation forming a chemotactic gradient for inflammatory and phagocytic cells to follow (Snyderman et al., 1970). Not only are C5a and C3a chemoattractants but also vasodilators, increasing the permeability of the microvasculature and facilitating effective cellular migration to sights of inflammation (Schumacher et al., 1991).

C5a is the most potent of the anaphylatoxins with multiple mechanisms of action (Frank and Fries, 1991). Two C5a receptors, known as C5aR and C5L2, have been identified (Cain and Monk, 2002; Gerard and Gerard, 1991). C5aR is a G-protein coupled receptor with seven transmembrane segments (Geva et al., 2000). C5aR is expressed on many cell types, including neutrophils, macrophages, monocytes, eosinophils, T cells and many other non-myeloid cells (Nataf et al., 1999; Zwirner et al., 1999).

Although similar in structure to C5aR (58% homology), the C5a receptor C5L2 is not G-protein coupled (Li et al., 2013). The exact function of C5L2 is a controversial topic. It was first thought to be a non-signalling, decoy receptor for C5a dampening inflammation by inhibiting C5aR signally (Okinaga et al., 2003). However, pro-inflammatory consequences of C5LR ligation have been described (Gao et al., 2005; Rittirsch et al., 2008). C5L2 also binds the inactive

anaphylatoxin fragment $C3a_{desArg}$ and $C5a_{desArg}$ with unknown functional consequences (Cain and Monk, 2002; Cui et al., 2009b).

C3a is roughly 50 to 100 times less potent that C5a (Ehrengruber et al., 1994; Fernandez et al., 1978). Only one receptor for C3a, known as C3aR, has been identified (Ames et al., 1996). Like C5aR, C3aR is a G-protein coupled receptor with seven transmembrane segments expressed on neutrophils, granulocytes and monocytes (but not T cells) (Martin et al., 1997).

Signalling through C5aR and C3aR changes the morphology of leukocytes (Servant et al., 2000). Cells quickly adopt a polarised morphology driven by actin polymerisation and the concentration of receptor involved in migration towards the leading edge of the cell driving migration upstream of the chemotactic gradient (Sadik et al., 2011).

1.3.2.3 Opsonisation

Deposition of complement proteins on the surface of pathogens targets them for and supports their phagocytosis (Aderem and Underhill, 1999; Frank and Fries, 1991; Wright and Silverstein, 1982). This process of labelling pathogens for phagocytosis is known as opsonisation. Opsonisation is particularly important for the clearance of pathogens resistant to CDC (Ram et al., 2010). Complement opsonins include C1q, C4b, MBL and the breakdown products of C3 cleavage (Bobak et al., 1987; Dunkelberger and Song, 2010; Kuhlman et al., 1989). Complement receptors (CRs) expressed by phagocytic cells, such as DCs, macrophages and neutrophils, bind complement opsonins initiating and enhancing phagocytosis (Fearon, 1980). At least five CRs are involved in effective phagocytosis: CR1, CR2, CR3, CR4 and CRIg (Ross and Medof, 1985).

CR1 and CR2 are structurally similar, consisting of an intracellular domain, transmembrane region and a long extracellular domain formed of 30 and 15 short consensus repeats (SCR), respectively (Ahearn and Fearon, 1989). Both receptors are members of the

family of regulators of complement activation which includes MCP, decay-accelerating factor (DAF), FH and C4 binding protein (C4BP) (Krych-Goldberg and Atkinson, 2001). CR1 exhibits the widest range of specificities of the receptors binding primarily to C3b and C4b but also MBL, C1q, iC3b and C3dg (Fearon, 1980; Ghiran et al., 2000; Klickstein et al., 1997; Krych-Goldberg and Atkinson, 2001). CR1 serves as a co-factor protein for FI and is necessary for the production of C3dg, an important molecule in reducing the antigen threshold needed for B cell activation (Dempsey et al., 1996; Medof et al., 1982; Nishida et al., 2006).

CR2 binds only iC3b, C3d and C3dg and, like CR1, serves as a co-factor protein for FI (Molina et al., 1994). Concurrent binding of the BCR and CR2 to antigens coated in C3dg significantly decreased the activation threshold in B cells (Lyubchenko et al., 2005; Nussenzweig et al., 1971). In B cells, CR2 associates with the membrane adapter protein CD19 which acts by recruiting SCR kinases (Vav, Lyn and PI 3-kinase) when co-ligated with BCR enhancing B cell activation (Fujimoto et al., 2000).

CR3 and CR4 are β2-integrins composed of CD18 and either CD11b or CD11c, respectively. CR3 and CR4 are specific receptors for iC3b (Ross and Větvička, 1993). Whilst both receptors are involved in phagocytosis of complement-tagged pathogens, CR3 functionally dominates (Sándor et al., 2013). CRIg is a member of the immunoglobulin superfamily which also includes CD19, CD4, CD8, antibodies and MHC classes I and II; specific for C3b and iC3b (Helmy et al., 2006).

1.3.3 Complement Regulation

Host regulators of the complement system come in two forms; those expressed on the cellular surfaces and those that are secreted into the serum (Zipfel and Skerka, 2009).

1.3.3.1 Soluble Complement Regulators

C4BP regulates the classical and lectin pathways of complement activation at the level of C3 convertase (Blom et al., 2001). C4BP is a large protein (570kDa) composed of eight subunits: seven identical α-chains with eight SCR domains and one β-chain with three SCR domains (Dahlbäck, 1983; Dahlbäck et al., 1983). The first three SCR domains of the α-chains bind C4b inhibiting the association with C2 and C3 convertase formation. C4BP has also been shown to accelerate the decay of the C3 convertase and act as a co-factor protein for FI (Gigli et al., 1979). It has also been suggested that C4BP binds C3b, functioning as a co-factor for FI (Blom et al., 2003).

FI is a serine protease regulating all three pathways by inactivating both C3b and C4b when they are bound to a co-factor protein (Masaki et al., 1992; Ross et al., 1982). Cofactor proteins for FI include FH, MCP, CR1 and C4BP (Lambris et al., 1996; Molina et al., 1994; Nishida et al., 2006). C3b cleavage by FI in the presence of the co-factors CR1, MCP or FH produces two fragments: iC3b, which remains membrane bound, and C3f, which is released into the circulation (Alcorlo et al., 2011). Cleavage disrupts the FB binding site thus removing the ability to form the C3 convertase of the alternative pathway. CR1 is the only co-factor able to facilitate further cleavage of iC3b between residues Arg^{932} and Glu^{933} by FI into C3c, which is released into the circulation, and C3dg, which remains anchored to the membrane (Furtado et al., 2008). C3dg is a ligand for CR2 involved in reducing the activation threshold in B cells when bound to BCR antigens (Dempsey et al., 1996; Lyubchenko et al., 2005). C4b cleavage by FI in the presence of the co-factors CR1, MCP and C4BP produces two fragments (iC4b and C4d) that are unable to

associate with C2 and form the C3 convertase of the classical and lectin pathways (Masaki et al., 1992).

Not only does FH possess co-factor activity for FI but decay accelerating activity against the C3 convertase of the alternative pathway (Wu et al., 2009). FH is a single-chain 155kDa plasma glycoprotein formed of 20 homologous SCRs connected to one another by short spacers of between three and eight amino acids in length (Kristensen et al., 1986; Nilsson and Müller-Eberhard, 1965; Ripoche et al., 1988; Whaley and Ruddy, 1976). The SCRs of FH are composed of approximately 61 amino acids which are each independently folded into globular structures consisting of six stranded antiparallel β-sheets by a series of loops and turns (Barlow et al., 1992; Okemefuna et al., 2009; Ripoche et al., 1988). Domains 1 and 2 are primarily responsible for the decay accelerator activity of FH which binds to the Bb binding region of C3b thus displacing Bb from the complex by steric hindrance and electrostatic repulsion. Domains 7, 19 and 20 anchors FH to glycosaminoglycans (GAGs) expressed on cell surfaces as well as membrane-bound C3b. Factor H-like protein (FHL-1) is a truncated form of FH composed of the first seven domains of FH that retains both co-factor activity for FI and decay accelerating activity for C3bBb (Zipfel and Skerka, 1999). The FH–related protein 1 (CFHR-1) is a truncated homologue of FH composed of only five SCR domains (de Jorge et al., 2013). The first two domains of CFHR-1 show 42% and 34% sequence homology with domains 6 and 7 of FH and the remaining three domains of CFHR-1 show 100%, 100% and 97% sequence homology with domains 18-20 of FH. Furthermore, CFHR-1 lacks domains 1-4 of FH responsible for the regulatory activity of C3 convertase but retains C3b and GAG binding through domains 4 and 5 (Heinen et al., 2009).

The serine proteases C1r and C1s, and MASP-1 and MASP-2 associate with the recognition molecules of the classical (C1q) and lectin (MBL) pathways, respectively (Lepow et al., 1963; Matsushita and Fujita, 1992; Thiel et al., 1997). Once activated, these serine proteases cleave C4 initiating the activation of both pathways (Héja et al., 2012; Müller-Eberhard et al.,

1967). The activity of these serine proteases is regulated by the protease inhibitor C1-inhibitor (C1INH) (Arlaud et al., 1979; Davis et al., 2010; Ratnoff and Lepow, 1957). C1INH is a member of the serine protease inhibitor (serpin) superfamily which also includes angiotensinogen and antitrypsin (Law et al., 2006). The substrate list for C1INH is not limited to complement proteases but includes several involved in the coagulation system, the fibrinolytic system and the plasma kallikrein–kinin system (Davis et al., 2008). C1INH becomes activated upon contact and cleavage of the peptide bond (Arg^{444}-Thr^{445}) in the reactive centre of C1INH by the C1q or MBL associated serine proteases. Cleavage of the peptide bond results in a conformational change of C1INH and generation of a covalent bond with the active site of the now inactivated, deformed serine protease (Huntington et al., 2000).

Plasma proteins clusterin and vitronectin are both negative regulators of complement that inhibit the formation of MAC and subsequent CDC of targeted cells (Tschopp et al., 1993). Both regulators have two modes of action: blocking the membrane binding site present in C7 in C5b-7 and preventing the incorporation of C9 into C5b-8 and C5b-9 (McDonald and Nelsestuen, 1997; Milis et al., 1993).

The activity of the anaphylatoxins C3a and C5a is regulated by the protease enzymes carboxypeptidase N and carboxypeptidase B (Bajic et al., 2013; Bokisch and Müller-Eberhard, 1970). C3a and C5a signal through the G-protein coupled receptors C3aR and C5aR, driving inflammation at sites of complement activation (Ames et al., 1996; Cain and Monk, 2002; Gerard and Gerard, 1991). C5a also binds to the receptor C5L2 although the exact consequences of this are unknown (Li et al., 2013). Carboxypeptidases cleave the C-terminal arginine residues of both C3a and C5a necessary for signalling through their respective receptors (Kaneko et al., 1995; Wilken et al., 1999). Although both carboxypeptidases show specificity for C3a and C5a, carboxypeptidase B shows a preference for C5a whereas carboxypeptidase N shows a

preference for C3a (Campbell et al., 2002). C5L2 also binds $C3a_{desArg}$ and $C5a_{desArg}$ (Cain and Monk, 2002; Cui et al., 2009a; Li et al., 2013).

Properdin is the only known positive regulator of the alternative pathway (Alcorlo et al., 2013; Hourcade, 2006). As previously discussed, properdin stabilises the C3 convertase C3bBb by binding to both C3b and Bb in the complex.

1.3.3.2 Membrane-bound Complement Regulators

CD59 is a glycosylphosphatidylinositol (GPI) anchored membrane glycoprotein able to bind both C8 (in C5b-8) and C9 (in C5b-9), disrupting the formation of MAC thus protecting cells from CDC (Meri et al., 1990). Binding of CD59 to C5b-8/9 complexes prevents the unfolding of C9 necessary for insertion through lipid membranes and additional polymerisation of C9 (Farkas et al., 2002).

The GPI-anchored membrane glycoprotein decay-accelerating factor (DAF; CD55) regulates the C3 convertases of the classical, lectin and alternative pathways (Nicholson-Weller et al., 1982). The extracellular region of DAF is composed of four complement control protein domains which bind to both C4b and C3b displacing C2a and Bb, respectively (Brodbeck et al., 2000). Classical and lectin pathway regulatory activity is contained with domains 2 and 3 whereas the alternative pathway regulatory activity is contained within domains 2, 3 and 4 (Brodbeck et al., 1996).

The FI co-factor activities of the membrane-bound complement regulators CR1 and MCP was previously described in ***Section 1.3.3.1***. CR1 also expresses decay accelerating activity for the C3 convertases of the alternative, classical and lectin pathways (Zipfel and Skerka, 2009). CR1 shows specificity for both C3b and C4b in each convertase displacing Bb and C2a, respectively.

1.3.4 Complement and Neisseria meningitidis

Complement-mediated lysis, facilitated by the MAC, is considered by some to be the most important mechanism in the protection against invasive meningococcal disease (Goldschneider et al., 1969a; Granoff, 2009). Opsonisation by products of complement activation and subsequent phagocytosis of *Neisseria meningitis* in protection is less well defined but thought to play a significant role in some individuals (Granoff, 2009). High incidence rates of meningococcal disease in those deficient in terminal pathway components (C5, C6, C7, C8 and C9) suggests the importance of bacteriolysis over opsonophagocytosis (Figueroa and Densen, 1991). *Neisseria meningitidis* has developed several mechanisms to evade the immune response ensuring effective colonisation of the host. The understanding of how *Neisseria meningitidis* manages to establish infection is of particular importance in the generation of potential targets for vaccination and prevention of disease. A summary of immune evasion mechanisms of *Neisseria meningitidis* is shown in ***Figure 1. 7***.

The FHbp is a 27kDa outer membrane lipoprotein expressed by all tested *Neisseria meningitidis* isolates that expresses an affinity for human FH and FHR-3 proteins (Caesar et al., 2014; Cendron et al., 2011; Madico et al., 2006). Sequence analysis of FHbp variants shows three (or two) distinct sub-families sharing at least 63% sequence similarity (Brehony et al., 2009; Murphy et al., 2009). FHbp is formed of two domains connected by a short loop: a curved eight-stranded antiparallel β-sheet at the N-terminal (containing region A and part of region B) and a eight-stranded antiparallel β-barrel at the C-terminal (containing the remainder of region B and region C) (Cantini et al., 2005; Cendron et al., 2011; Schneider et al., 2009; Serruto et al., 2012). Regions A, B and C of FHbp interact with FH SCRs 6 and 7 maintaining co-factor and decay accelerator properties of bound FH thus increasing the survival of *Neisseria meningitidis* in serum (Granoff et al., 2009; Madico et al., 2006; Schneider et al., 2009).

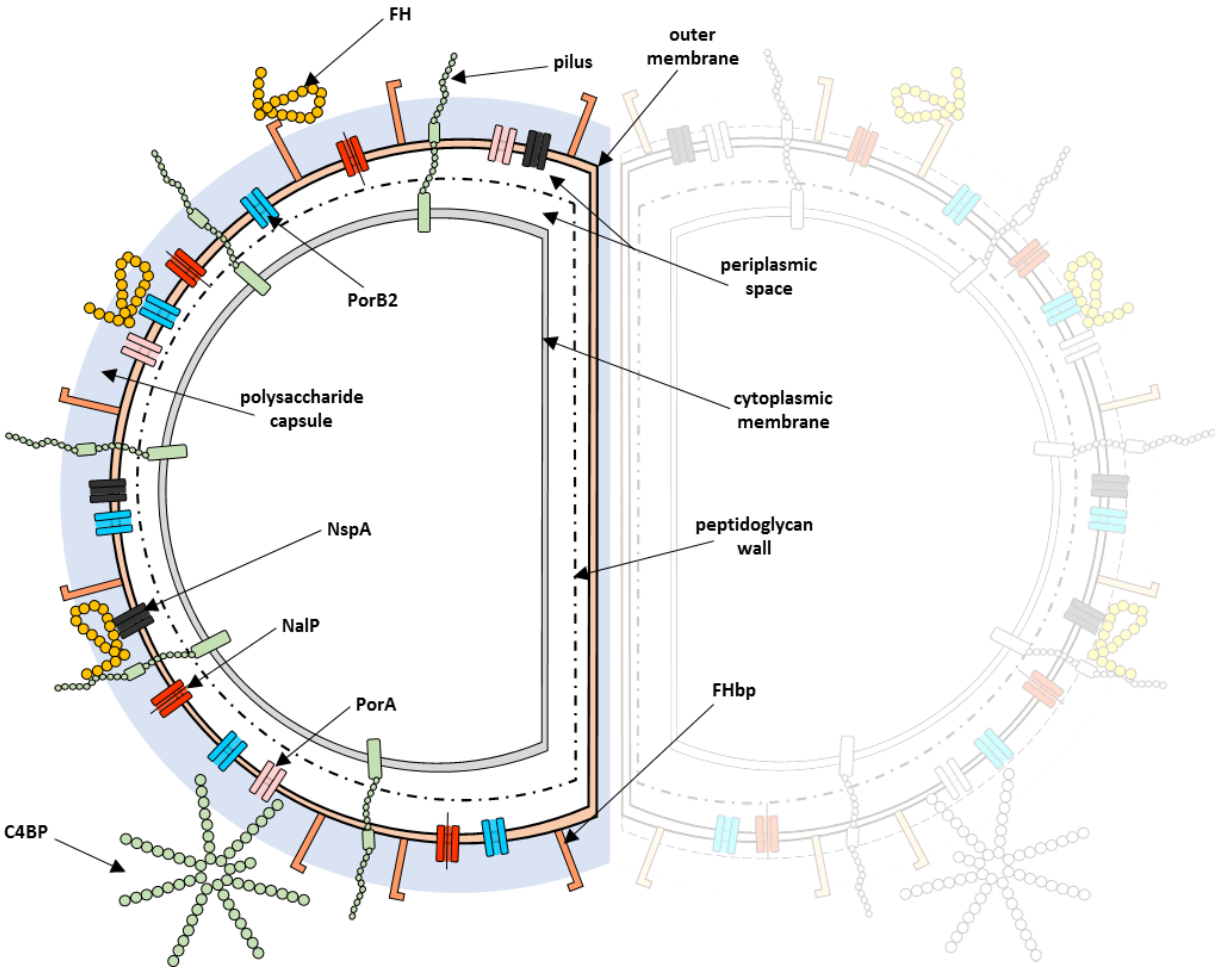


Figure 1. 7 Complement Evasion Mechanisms of *Neisseria meningitis*

Factor H binding protein (FHbp) recruits fluid-phase complement regulator FH to the surface of the bacteria inhibiting complement activation by cleaving of C3b, in the presence of Factor I, and accelerating the decay of the alternative pathway C3 convertase (C3bBb). Surface proteins PorB2 and NspA also bind FH. The porin PorA reduces complement-mediated lysis by binding C4BP, a co-factor for FI in the cleavage and inactivation of C4b. The polysaccharide capsule of *Neisseria* limits classical pathway activation by sterically hindering C1q engagement to the surface of antibody labelled bacteria. The auto-transporter NalP specifically cleaves human C3 into inactive C3a-like and C3b-like fragments, reducing anaphylatoxin release and further complement activation.

Other FH binding proteins identified on *Neisseria meningitidis* include porin B2 (PorB2), PorB3 and *Neisserial* surface protein A (NspA) (Giuntini et al., 2015; Lewis et al., 2010; Lewis et al., 2013). The auto-transporter protease NalP specifically cleaves human C3 into an inactive C3b-like fragment and a C3a-like fragment (Del Tordello et al., 2014). Although the C3a-like fragment was not functionally assayed, structural analysis revealed the fragment lacked the C-terminal arginine thought to be essential for activity (Bokisch and Müller-Eberhard, 1970). The porin PorA expressed on the surface of the bacterium binds the complement inhibitor C4BP. C4BP regulates the classical and lectin pathways of complement activation at the level of C3 convertase (Jarva et al., 2005).

Each pathway of complement activation has been implicated in the protection against meningococcal disease. The relative importance of each of the three pathways of complement activation has been assessed using serum deficient in individual components or pathway-specific inhibitors.

1.3.4.1 Neisseria meningitidis *and The Classical Pathway*

The classical pathway of complement is activated by C1q binding primarily to antigen bound immunoglobulin (Cooper, 1985). The presence of bactericidal antibody specific for *Neisseria meningitidis* is a well-defined correlate of protection as previously discussed. Although not nearly as pronounced as terminal pathway deficiency, C2 deficiencies are associated with an increased risk of meningococcal disease (Ross and Densen, 1984). However, vaccination against *Neisseria meningitidis* does produce strong SBA titres in individuals deficient in C2.

Direct C1q binding and classical pathway activation on Gram-negative bacteria has been described (Mintz et al., 1995; Tenner et al., 1984); however, the benefit of the classical pathway in the absence of bactericidal antibody is minimal (Agarwal et al., 2014; Drogari-Apiranthitou et

al., 2002). In a recent study, some classical pathway activation was seen on *Neisseria meningitidis* mutants lacking capsules highlighting the polysaccharide capsule as an important virulence factor for invasive disease (Agarwal et al., 2014). Encapsulation of the bacteria abolished all C1 activation, which was restored by anti-*Neisseria meningitidis* antibody.

1.3.4.2 Neisseria meningitidis *and The Lectin Pathway*

The lectin pathway is initiated by MBL (Ikeda et al., 1987; Kawasaki et al., 1978; Wild et al., 1983). MBL binds a number of ligands, including those expressed on the surface of *Neisseria meningitis* (Estabrook et al., 2004; Sprong et al., 2004). Deficiency in MBL is associated with increased meningococcal disease in infants (Hibberd et al., 1999; Jack et al., 1998; Jack et al., 2001; van Helden and Hoal-van Helden, 1999). It was first thought that the ligand for MBL on *Neisseria meningitis* was lipooligosaccharides and that level of sialylation of lipooligosaccharides dictates MBL binding (Estabrook et al., 1997; Jack et al., 1998; Jack et al., 2001; Mackinnon et al., 1993; Van Emmerik et al., 1994). It was later shown that MBL binds to both meningococcal opacity (Opa) and PorB proteins and complement activation via the lectin pathway is now considered somewhat independent of lipooligosaccharide (Estabrook et al., 2004; Sprong et al., 2004). PorB protein also binds FH (Giuntini et al., 2015; Lewis et al., 2013).

The bactericidal activity of the lectin pathway towards *Neisseria meningitis* is unclear. In one study, no difference in the bactericidal activity was seen between MBL deficient and sufficient serum (Hellerud et al., 2010). In contrast, other studies have shown significantly reduced complement activation when the lectin pathway was inhibited or absent (Jack et al., 2001; Sprong et al., 2003). However, blockade of the alternative pathway abolished almost all complement deposition on the bacteria despite an intact lectin pathway showing the dependence of lectin pathway activation on amplification by the alternative pathway (Sprong et al., 2003). These data suggest that the lectin pathway can be activated by *Neisseria meningitis*

but at such low levels as to not contribute significantly to either direct killing by MAC formation or clearance by opsonophagocytosis (Drogari-Apiranthitou et al., 1997). Nevertheless, the importance of the lectin pathway in disease prevention in more vulnerable individuals may be argued (Hibberd et al., 1999; van Helden and Hoal-van Helden, 1999).

1.3.4.3 Neisseria meningitidis *and The Alternative Pathway*

Alternative pathway component deficiencies, genome-wide association studies and interactions of *Neisseria meningitidis* with components of the alternative pathway all indicate the importance of the alternative pathway in protection against meningococcal disease (Consortium, 2010; Figueroa and Densen, 1991; Madico et al., 2006).

Single nucleotide polymorphisms present in genes coding for FH and FH-related protein 3 (FHR-3) were associated with increased susceptibility to meningococcal disease in a recent genome-wide association study (Consortium, 2010). The serum protein FHR-3 is a truncated homologue of FH lacking SCRs 1 to 4 and thus has no co-factor and decay accelerator activity (Skerka et al., 2013). Additional examination on the specificity of FHbp showed somewhat indiscriminate binding between FH and FHR-3 with SPR analyses showing that the equilibrium dissociation constants (KD) of FHbp to human FH and FHR-3 are similar; ranging between $2.0e^{-9}$ and $7.2e^{-9}$ M (Caesar et al., 2014; Johnson et al., 2012; Schneider et al., 2009). Both FH and FHR-3 compete for FHbp binding and thus, relative serum levels of each either increase susceptibility to or protect against disease. Caesar et al., (2014) showed that the survival of *Neisseria meningitidis* in human serum is significantly reduced when the bacteria are pre-incubated with full length FHR-3 due to a reduction in FH binding to FHbp. Increased rates of disease are seen in individuals deficient in FD or properdin further supporting the importance of the alternative pathway in the clearance of *Neisseria meningitidis* (Figueroa and Densen, 1991; Linton and Morgan, 1999; Morgan and Walport, 1991).

Whilst the alternative pathway in conjunction with the classical pathway of complement activation is considered crucial in the protection against meningococcal, the direct contribution of the alternative pathway to bactericidal activity is less well characterised (Hellerud et al., 2010; Ram et al., 2011). In the absence of bactericidal antibody or in the presence of lectin and classical pathway inhibitors, active components of the alternative pathway can be found on all serogroups following incubation with serum (Bjerre et al., 2002; Ram et al., 2011; Sprong et al., 2003). The structure of the polysaccharide capsule is thought to regulate alternative pathway activity. For serogroups C and B, strains expressing polysaccharide capsules containing sialic acid inhibit activation of the alternative pathway and strains resistant to bactericidal killing often have very high capsule expression (Hellerud et al., 2010; Jarvis and Vedros, 1987; Uria et al., 2008). This resistance is thought to be driven by the masking of lacto-N-neotetraose present in the LPS of *Neisseria meningitidis* by sialic acid, reducing C3b binding (Estabrook et al., 1997). Conversely, capsule expression of serogroups W and Y has been shown to enhance alternative pathway activation by increased interaction with Gal and Glc residues and C3 (Ram et al., 2011). This may explain why properdin and FD deficient patients are more susceptible to rarer serogroups such as W and Y (Figueroa and Densen, 1991; Morgan and Walport, 1991). In this same study, C2 deficient serum did not kill any of the strains tested stressing the importance of the classical pathway in bacterial clearance. In conclusion, direct complement activation on *Neisseria meningitidis* by the alternative pathway is highly variable and limited.

1.4 Complement Source in Serum Bactericidal Assays

Following the successful development of protective meningococcal vaccines against serogroups A and C in the 1970s, the World Health Organisation (WHO) published several recommendations to public health organisations in regards to standardising the production, storage and testing of these vaccines (WHO, 1976). In the absence of an appropriate animal model to assess the potential of new meningococcal vaccines in humans, it was proposed that vaccine efficacy must be tested by measurement of bactericidal antibody titres before and 2-4 weeks post vaccination. Bactericidal antibody titres are measured in vaccinee serum by SBA as described in ***Section 1.1.5.1***. The bactericidal titre of vaccinee serum is calculated as the dilution of serum that results in 50% or greater killing of the target bacteria after a one-hour incubation with a source of complement. For a particular vaccine to be considered effective, more than 90% of individuals must have a greater than 4-fold rise in bactericidal titre and bactericidal titre of at least 4. These recommendations were based on the findings by Gotschlich, Goldschneider and Artenstein who demonstrated the correlation between bactericidal antibody and protection against invasive meningococcal disease (Goldschneider et al., 1969a; Goldschneider et al., 1969b; Gotschlich et al., 1969a). Several amendments to these recommendations by WHO have been put forward including considerations for additional correlates of protection (WHO, 2004, 2006). These include the measurement of anti-meningococcal antibody concentration and avidity post vaccination (Borrow et al., 2001a; Gheesling et al., 1994; Granoff et al., 1998; Jodar et al., 2000). However, the SBA still remains the gold standard correlate in the assessment of new meningococcal vaccines for licensure.

Despite these recommendations from the WHO on the standard protocol for the SBA, many laboratories continued to use different methods when assessing meningococcal vaccines (Frasch and Robbins, 1978; Mandrell et al., 1995; Maslanka et al., 1997; Ross et al., 1985; Söderström et al., 1987; Zollinger et al., 1988; Zollinger and Mandrell, 1983). Variations in assays

(including complement source and concentration, buffers, incubation times and growth of the target strain of bacteria) made the inter-laboratory assessment of new vaccines impossible. In response, a consortium of researchers in the field evaluated each variation of the SBA to create a standardised and reproducible assay (Maslanka et al., 1997). The SBA requires a source of complement lacking antibody specific to the target bacteria to measure the true bactericidal titre of an individual. Both human and BRS were assessed for their use in the SBA. Although human serum would be the ideal source of complement for these assays, the high prevalence of naturally acquired or vaccine-induced bactericidal antibody present would make finding a suitable donor very difficult indeed. In contrast, BRS was found to be a suitable and convenient reagent showing little batch-to-batch variation, minimal background bactericidal activity to certain strains of serogroup A and C in the absence of antibody and a reasonable correlation between SBA titres and anti-meningococcal antibody concentration.

As noted in previous and subsequent studies, SBA titres achieved with BRS are significantly higher than with human serum (Borrow et al., 2001a; Jodar et al., 2000; Maslanka et al., 1997; Zollinger and Mandrell, 1983). An even greater concern is the poor correlation between SBA titres with human serum and BRS, especially for serogroups A, W and Y (Findlow et al., 2009; Gill et al., 2011a). Interestingly, SBA titres between both sources of serum for serogroup C isolates tend to correlate well. The average correlation (R-squared values ± standard deviation) between SBA titres from two individual studies for serogroups A, C W and Y was 0.34 ± 0.14, 0.73 ± 0.04, 0.56 ± 0.01 and 0.49 ± 0.78, respectively (Gill et al., 2011a). These data have questioned the validity of using BRS in SBAs as a correlate of protection in trials assessing the efficacy of meningococcal vaccines for licensure (WHO, 2006). As will be discussed below and in subsequent chapters, several hypotheses have been proposed for the differences seen between human serum and BRS in SBAs.

1.4.1 Differences in the Interaction of Human and Rabbit Complement with Neisseria meningitidis

Neisseria meningitidis employs a number of techniques to aid survival in the circulation by regulating complement activation (Agarwal et al., 2014; Caesar et al., 2014; Del Tordello et al., 2014; Giuntini et al., 2015; Jarva et al., 2005; Lewis et al., 2010; Lewis et al., 2013; Madico et al., 2006; Uria et al., 2008). These mechanisms include sequestering host regulators of complement (such as C4BP and FH), cleavage and inactivation of C3 and evasion of recognition by capsule expression containing high levels of sialic acid. Expression of many of these complement regulators is upregulated by increases in host temperature; a common response to infection (Loh et al., 2013). *Neisseria meningitidis* is a human only pathogen and no other animal reservoirs have been identified. It may be hypothesised that complement regulation by *Neisseria meningitidis* is restricted to human complement only which may account for the higher SBA titres seen with BRS. In fact, this species-specific complement regulation has been described for *Neisserial* proteins FHbp and NalP (Del Tordello et al., 2014; Granoff et al., 2009). NalP cleaves human C3 into non-functional C3a-like and C3b-like fragments increasing survival in serum but did not cleave either mouse or rabbit C3 (Del Tordello et al., 2014). FHbp binds the SCRs 6 and 7 of human FH, maintaining co-factor factor activity for FI and decay accelerator activity towards C3bBb, increasing the survival in serum, but does not bind rabbit or rat FH (Granoff et al., 2009). Whether the other mechanisms of complement evasion are restricted to human complement has not been investigated. However, based on previous data it is most likely that these mechanisms do not regulate rabbit complement.

1.4.2 Activation of Human and Rabbit Complement by Human Immunoglobulins

Whilst differences between the interaction of human and rabbit complement and complement regulators with *Neisseria meningitidis* may explain why SBA titres with BRS are much higher than with human serum, these species-specific differences are presumably constant and do not provide an adequate explanation for the poor correlation between hSBA and rSBA titres seen for some serogroups. In these assays, bacterial strains and complement sources are constant. The only variable in these assays are the vaccinee sera which may vary in the magnitude and type of antibody response to vaccine antigens. As such, it may be hypothesised that differences in antibody response to vaccine antigens and the ability of anti-meningococcal antibodies to activate human and rabbit complement may account for the poor correlation between SBAs with human serum and BRS.

Differences in the ability of human immunoglobulins to activate human and rabbit complement and their impact on SBA titres is poorly understood. Several studies have shown that 500-1000 times less human anti-meningococcal IgM antibody is required to kill *Neisseria meningitidis* in the presence of BRS than with human serum in SBA whilst only 10 times less human anti-meningococcal IgG antibody is required (Griffiss and Goroff, 1983; Mandrell et al., 1995; Santos et al., 2001; Zollinger and Mandrell, 1983). Furthermore, it has been shown that human IgG1 and IgG3 activates human complement better than rabbit complement whilst human IgG2 activates rabbit complement better than human (Dangl et al., 1988). Human IgG4 does not activate either human or rabbit complement. The mechanisms behind these differences and their impact on SBA titres with either human serum or BRS is unknown. However, these data suggest that a primarily IgM and IgG2 response to meningococcal vaccines will produce misleadingly high SBA titres with BRS whilst a predominantly IgG1 and IgG3 antibody response will produce a misleadingly low SBA titre with BRS.

1.5 Study Aims

The purpose of this project is to further evaluate the differences between rabbit and human complement with regards to interactions with human immunoglobulins and *Neisseria meningitidis*; as one component of this, the antibody subclass response to meningococcal polysaccharide vaccination will be explored, in particular how this impacts on SBA titres with human serum and BRS. The specific aims of this project are as follows:

i. To establish whether there are differences in interactions between human IgG subclasses and IgM with the vaccine and rabbit and human C1q.
ii. Investigate which subclasses of antibodies are most strongly induced by conjugated and plain polysaccharide meningococcal vaccine and whether the nature of this response varies with age.
iii. Compare whether human IgG subclasses and IgM differentially activate rabbit and human complement.
iv. Assess the contribution of the different IgG subclasses and the various complement activation pathways to bactericidal killing with rabbit or human complement.

Data from this project will help inform a rational approach to selecting a complement source for clinical trial serology for regulatory approval of new vaccines and ultimately assist improvements in the development and testing of meningococcal vaccines in the future.

Chapter Two – Materials and Methods

2.1 Preparation of Plasma and Serum

Human blood samples were obtained via withdrawal from the median cubital vein by syringe draw with a 21-gauge butterfly needle. Fresh rabbit blood was obtained via withdrawal from either the marginal ear vein by syringe draw with a 21-gauge butterfly needle or by cardiac puncture, the latter under terminal anaesthesia. Frozen rabbit serum was acquired from VH Bio Limited, Gateshead, UK.

For serum, whole blood samples were left to clot for 30 minutes at ambient temperature. Clots were contracted on ice for 30 minutes. Samples were then centrifuged at 3500RPM (2671g) for 30 minutes at 4°C. Serum was aspirated from the clot and re-centrifuged at 3500RPM for 30 minutes at 4°C. Serum was aspirated, syringe filtered through a 0.22µm, 33mm, non-sterile Millex-GP filter (Millipore Limited, Hertfordshire, UK) and stored at -80°C. If required, complement was heat-inactivated by incubation of serum at 56°C for 30 minutes in a water bath with gentle manipulation every 5-10 minutes.

Plasma was prepared as with serum but with the addition of 1mL 0.5M ethylenediaminetetraacetic acid, pH7.4 (EDTA; Sigma-Aldrich Company Ltd, Dorset, UK) per 50mL whole blood at time of collection to prevent clotting. Plasma samples were stored in aliquots at -20°C.

2.2 Fast Protein Liquid Chromatography (FPLC)

All FPLC work was performed at 4°C using an ÄKTApurifier 10 chromatography machine (GE Healthcare Life Sciences, Buckinghamshire, UK) with UNICORN Control Software (GE Healthcare Life Sciences). All buffers were vacuum filtered through a 0.2µm cellulose acetate membrane (Millipore) before use.

2.2.1 Amine-Coupling to NHS-activated Sepharose™ Columns

Ligands were amine-coupled to 1mL and 5mL HiTrap N-hydroxysuccinimide- (NHS) activated Sepharose™ High Performance (HP) columns as per manufacturer's instructions (GE Healthcare Life Sciences). Briefly, six column volumes (CVs) of ice-cold 1mM HCl was injected over the column to wash out the isopropanol used to preserve active esters. Ligands were concentrated to 5-10mg/mL using an Amicon (R) Ultra 4mL Centrifugal Filter Unit with Ultracel 100kDa membrane (Millipore Limited) and dialysed overnight at 4°C in 0.2M $NaHCO_3$ (Sigma), 0.5M NaCl, pH 8.3 (coupling buffer; CB). One CV of concentrated, equilibrated ligand was injected into the column and incubated at ambient temperature for 30 minutes.

After coupling, the column was washed with CB (3 CV) and fractions collected to measure unbound protein and enable coupling efficiency to be calculated. Residual active esters were then deactivated by washing with six CV of 0.5M ethanolamine, 0.5M NaCl, pH 8.3 (buffer A) followed by six CV of 0.1M sodium acetate, 0.5M NaCl, pH 4 (buffer B). This wash cycle was repeated three times.

2.2.2 Human IgM and Human and Rabbit IgG Antibody Isolation

Human IgM was isolated from plasma using a CaptureSelect™ IgM Affinity Matrix (Life Technologies, Paisley, UK). CaptureSelect™ IgM Affinity Matrix contains the light chain fragment of a monoclonal antibody specific for the Fc region of IgM which is amine coupled to NHS-activated Sepharose 4 Fast Flow beads (GE Healthcare). The matrix was packed into a Proteus 5mL FliQ™ Column (Generon Ltd., Berkshire, UK). The column was pre-equilibrated in PBS and 5CV human plasma injected. Unbound proteins were washed from the column with PBS until effluent absorbance (280nm) was below 2mAU and bound IgM antibody was eluted with 3CV 0.1M glycine-HCl pH2.5 whilst collecting 1mL fractions. Eluted IgM was immediately neutralised with a solution of 1M Tris pH8.0 (1mL per 5mL eluted protein), poured in cellulose dialysis tubing and dialysed against at least 50 volumes of PBS overnight at 4°C.

Human and rabbit IgG was isolated by injection of plasma over a 1mL pre-packed Protein G column (GE Healthcare Life Sciences). The column was pre-equilibrated in PBS and 5CV human plasma injected. Unbound proteins were washed and bound IgG was eluted and prepared as described above.

2.2.3 Human Anti-MenACWY Antibody Isolation

Meningococcal serogroup A, C, W and Y-specific anti-polysaccharide antibody (anti-MenACWY) was isolated by injecting plasma sequentially over two 5mL HiTrap NHS-Activated HP columns. The first column had 40.6mg tetanus toxoid (TT; GlaxoSmithKline (GSK) Biologicals SA, Wavre, Belgium) amine-coupled and the second had 25.2mg MenACWY-TT (GSK Biologicals SA) coupled. Plasma was first injected over the TT column to deplete anti-TT antibody before injection over the MenACWY-TT column to isolate polysaccharide-specific antibody. Unbound proteins were washed from each column with PBS (280nm<2mAU) and bound anti-TT (TT column) and anti-MenACWY (MenACWY-TT column) antibodies were separately eluted with

3CV 0.1M glycine-HCl pH2.5 whilst collecting 1mL fractions. Eluted antibodies were immediately neutralised with a solution of 1M Tris pH8.0 (1mL per 5mL eluted protein) and dialysed against at least 50 volumes of PBS overnight at 4°C.

2.2.4 Human IgG1, IgG2, IgG3 and IgG4 Antibody Separation

Human plasma or purified antibody was first injected over an anti-human IgG2 and an anti-human IgG4 column connected in series. The anti-human IgG2 column was generated by amine coupling 5mg of a monoclonal anti-human IgG2 antibody (Stratech Scientific Limited, Suffolk, UK) to a 1mL HiTrap NHS-Activated HP column. The anti-human IgG4 column was generated by packing 5mL of CaptureSelect™ IgG4 Affinity Matrix (Life Technologies) into an empty XK chromatography column (GE Healthcare Life Sciences). CaptureSelect™ IgG4 Affinity Matrix contains the light chain fragment of a monoclonal antibody specific for the Fc region of IgG4 which is amine coupled to NHS-activated Sepharose 4 Fast Flow beads (GE Healthcare). After loading, each of the columns were washed with PBS (280nm<2mAU) and separately eluted with 3CV 0.1M glycine-HCl pH2.5 whilst collecting 1mL fractions. Eluted antibodies were immediately neutralised with a solution of 1M Tris pH8.0 (1mL per 5mL eluted protein), dialysed against at least 50 volumes of PBS overnight at 4°C and concentrated to 1mg/mL. To ensure complete depletion, the plasma was injected over each column several times.

Human IgG1 and IgG3 antibodies were isolated based on their differential binding affinities for Protein G and Protein A (Akerström and Björck, 1986; Kronvall and Williams, 1969). Protein A binds the Fc region of IgG1, IgG2 and IgG4 but not IgG3 whereas Protein G binds all human IgG subclasses. One CV of the IgG2 and IgG4 depleted human plasma was injected over a 1mL Protein A column (IgG1), and a 1mL Protein G column (IgG3); the columns were connected in series. Both columns were washed in series with PBS and eluted individually as above. Eluted antibodies were prepared as described above.

2.2.5 Human C1q Isolation

Human C1q was isolated using the protocol first described by Tenner et al., with minimal modification (Tenner et al., 1981). Approximately 80mL, human serum containing 0.01M EDTA was injected through BioRex 70 resin (100-200 mesh size; Bio-Rad Laboratories Ltd., Hertfordshire, UK) packed into an empty XK chromatography column (GE Healthcare Life Sciences;) equilibrated with phosphate buffer pH 7.4 containing 83mM NaCl, 0.01M EDTA; after loading, the column was washed in the same buffer to baseline UV. BioRex70 cation exchange resin contains carboxylic acid exchange groups which facilitate the electrostatic interaction with human C1q. The CV was roughly 40mL. Bound proteins were eluted with phosphate buffer pH 7.4 containing 300mM NaCl, 0.01M EDTA. The protein-containing fractions were pooled and an equal volume of saturated ammonium sulphate solution was added drop-wise to the eluted proteins at 4^{o}C. This solution was gently mixed for 30 minutes at 4°C and allowed to fully precipitate overnight at 4^{o}C. The resultant suspension was spun at 8000RPM (6105g) for 20 minutes at 4°C. The supernatant was poured away and pellet dissolved in 500µL 0.5M NaCl, 0.05M Tris pH7.4, 0.01M EDTA. The dissolved pellet was 0.2uM filtered then 200µL was applied to a 24mL Superose 6 gel filtration column (GE Healthcare) to remove aggregates. The main protein peak containing monomeric C1q was pooled and applied to a 1mL Protein L column (GE Healthcare) pre-equilibrated with 0.01M HEPES pH 7.4, 0.15M NaCl, 3mM EDTA, 0.005% v/v surfactant P20, to remove contaminating immunoglobulins. The breakthrough protein was pooled and stored at -80°C.

2.2.6 Rabbit C1q Isolation

Rabbit C1q was isolated as described by Pohl et al. (1980), with some modification. Rabbit anti-human IgG serum was generated as described in ***Section 2.3***. The immune serum (80mL), containing 0.01M EDTA, was injected over a 5mL human IgG column, pre-equilibrated with PBS 0.01M EDTA. The human IgG column was generated by amine coupling 44.8mg purified human IgG antibody to a 5mL HiTrap NHS-Activated HP column. In this way, an immune complex (anti-human rabbit IgG bound to human IgG) was generated on the column creating an appropriate surface for rabbit C1q to bind.

After injection, weakly bound proteins were removed from the column without disturbing the immune complex by washing to baseline with 1M NaCl, 10mM phosphate pH7.4. Non-immune rabbit serum (3x100mL) was then injected over the immune column, pre-equilibrated with PBS 0.01M EDTA. After loading, the immune complex column was washed with PBS 0.01M EDTA (280nm<2mAU) and bound C1q was eluted with 3CV 1M NaCl, 10mM phosphate pH7.4. The eluted C1q was concentrated to 5mg/mL using an Amicon Ultra 4mL Centrifugal Filter Unit with Ultracel 100kDa membrane (Millipore) and 200µL was applied to a 24mL Superose 6 gel filtration column to remove aggregates. The main protein peak containing monomeric C1q was pooled and applied to a 1mL Protein L column (GE Healthcare) pre-equilibrated with 0.01M HEPES pH 7.4, 0.15M NaCl, 3mM EDTA, 0.005% v/v surfactant P20, to remove contaminating immunoglobulins. The breakthrough protein was pooled and stored at -80°C.

2.2.7 Human FH Isolation

Human FH was isolated from plasma by a two-step affinity chromatography method followed by size exclusion chromatography. Human plasma (4x15mL) was injected over a 5mL HiTrap NHS-Activated HP column to which 10mg anti-human FH antibody (MBI-7) was coupled (Hakobyan et al., 2008). The column was washed with PBS and bound protein eluted with 3CV 0.1M glycine-HCl pH2.5. Eluted protein was dialysed into 50 times volume of 150mM NaCl, 20mM Tris, pH7.4 and applied to a 1mL HiTrap™ Heparin HP column (GE Healthcare Life Sciences) pre-equilibrated with 150mM NaCl, 20mM Tris, pH7.4. The column was washed to baseline with 150mM NaCl, 20mM Tris, pH7.4 and eluted with 1M NaCl 20mM Tris pH7.4. The heparin column protein eluate was then injected over Superdex-200 matrix (GE Healthcare Life Sciences) in a XK16/70 column (GE Healthcare Life Sciences) pre-equilibrated in PBS in order to remove aggregates. The FH-containing fractions were pooled, concentrated to 1mg/mL and stored in aliquots at -20°C.

2.3 Rabbit Immunisation with Human IgG Antibody

Non-specific human IgG (isolated from plasma as described in ***Section 2.2.2***) was diluted to 600µg/mL in PBS and mixed thoroughly 1/1 (v/v) with Freund's Complement Adjuvant (Sigma) to create a stable emulsion. A New Zealand White rabbit (*Oryctolagus cuniculus*) was immunised by subcutaneous injection with 1mL of the human IgG-containing emulsion over at least four sites on the flanks. The rabbit was boosted at 4, 5 and 6 weeks after first injection with 1mL of an emulsion containing 600µg/mL human IgG made with Freund's Incomplete Adjuvant (Sigma). One week after the final immunisation, the rabbit was euthanised by cardiac puncture and exsanguination under terminal anaesthesia.

2.4 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Polyacrylamide gels were self-cast in a four gel caster for 10x8cm plates (Hoefer Inc. Massachusetts, USA) by pouring 25-30mL resolving gel solution between a sandwich of a glass and aluminium plate either side of a 0.75mm spacer (Hoefer Inc.) leaving a 2-4cm air gap above the gel. To prevent the resolving gel from drying out whilst setting and to remove air bubbles, 1mL of 70% (v/v) ethanol (Fisher Scientific UK Ltd, Loughborough, UK) was pipetted over the top of the resolving gel carefully, so as to not disturb the gel layer. The resolving gel solution was made up of 7-12mL 40% (w/v) acrylamide (Fisher Scientific UK Ltd), 8mL 1.5M Tris pH 8.8, 320µL 10% (w/v) SDS, 320µL 10% (w/v) ammonium persulfate (Sigma) and 32µL tetramethylethylenediamine (TEMED; Bio-Rad Laboratories Ltd). Once set, the ethanol was poured away and stacking gel solution poured on top of the resolving gel to the top of the cast. A 10-15 well 0.75mm comb (Hoefer Inc.) was inserted between the glass and aluminium plates into the stacking gel before the gel had set. Stacking gel solution was made up 2mL 40% (w/v) acrylamide, 5mL 0.5M Tris pH 6.8, 200µL 10% (w/v) SDS, 200µL 10% (w/v) ammonium persulfate and 20µL TEMED.

Gel casts, held in a mini-vertical gel electrophoresis unit with spring clamps (Hoefer Inc.), were flooded with SDS-PAGE running buffer composed of 25mM Tris, 192mM glycine (Fisher Scientific UK Ltd) and 0.1% (w/v) SDS. Between 10-20µL/well of sample, diluted in PBS to 0.1-1mg/mL, was loaded between the glass and aluminium plates of the gel mould (Hoefer Inc.). Prior to loading, each sample was diluted 5/1 (v/v) with non-reducing or reducing loading buffer and heated between 90-100°C for two minutes using a dry block heater. Loading buffer was composed of 375mM Tris, 12% (w/v) SDS, 60% (v/v), glycerol (Fisher Scientific UK Ltd), 0.06% (w/v) bromophenol blue (Bio-Rad Laboratories Ltd) with (reducing) or without (non-reducing) 5% (v/v) β-mercaptoethanol. Between 5µL and 10µL of a pre-stained protein marker set, 7-

175kDa or 10-190kDa, was run alongside samples to assess size and progression through the gel (New England Biolabs Inc, Massachusetts, USA). Once loaded, the gel electrophoresis unit was connected to a 300 volt power pack (VWR International, Leicestershire, UK) and run for 20 minutes at 120V followed by 1-2 hours at 180V. The gel electrophoresis unit was plumbed into a cooling water pump and cooled to 5°C to prevent overheating and the gel from drying out.

2.4.1 Coomassie Stain

Gels were submerged in a solution of 40% (v/v) methanol (Fisher Scientific UK Ltd), 10% (v/v) acetic acid (Fisher Scientific UK Ltd) and 0.5% (w/v) R250 Brilliant Blue (Life Technologies Ltd) for one hour at ambient. Gels were destained with 40% (v/v) methanol, 10% (v/v) acetic acid overnight at ambient temperature with gentle agitation. Once sufficiently de-stained, gels were submerged in a solution of 20% (v/v) ethanol, 10% (v/v) glycerol for one hour at ambient temperature and imaged with a myECL™ Imager (Thermo Fisher Scientific, Massachusetts, USA). Gels were dried for preservation between two sheets of gel drying film (Promega, Southampton, UK) held within a 20cm x 17.5cm gel drying stage (Promega).

2.4.1.1 Calculation of Molecular Weight of Purified Proteins

A plot of relative migration distance (R_f) and log molecular weight of each protein of the molecular weight marker was generated using GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego California USA). R_f was calculated as the distance migrated (cm) by the protein through the gel over the distance migrated by the dye front. Distance migrated was measured from the top of the resolving gel. The molecular weight for each protein of unknown weight was then interpolated from their respective R_f values using the molecular weight marker as a standard.

2.4.2 Western Blot

Gels were submerged in Pierce™ 1-Step Transfer Buffer (Thermo Fisher Scientific) for 30 minutes at ambient. The exact composition of Pierce™ 1-Step Transfer Buffer is not published but contains approximately 336mM tris, about 260mM glycine, about 140mM tricine, and if EDTA is used, approximately 2.4mM EDTA (Patent Number US20140224655 A1). Four sheets per gel of chromatography paper (10x8cm; Whatman™ 3MM Chromatography Paper) and one sheet per gel of nitrocellulose membrane (Amersham Protran Supported 0.45µm) of the same size were also submerged in Pierce™ 1-Step Transfer Buffer for 30 minutes at ambient. Protein was then transferred from the gel onto the nitrocellulose membrane using a Thermo Scientific™ Pierce™ Power Blotter as instructed. Once transferred, membranes were submerged in a solution of PBS, 0.1% (v/v) Tween 20 (Thermo Fisher Scientific), 5% (w/v) milk (Marvel, blot blocking buffer) for one hour at ambient temperature or overnight at 4°C on a roller. Blot blocking buffer was poured off and membranes were incubated with a primary detection antibody diluted 1/400 to 1/1000 (v/v) in blot blocking buffer for one hour at ambient temperature on a roller. A list of antibodies used in western blot assays are shown in ***Table 2.1***. Membranes were then washed with PBS, 0.1% (v/v) Tween 20 for five minutes, three times. If a secondary detection antibody was required, membranes were incubated with the secondary antibody as above. Once sufficiently washed, 1-2mL/membrane of Amersham ECL Western Blotting Detection Reagents one and two (mixed 1:1 (v/v) immediately before use; GE Healthcare Life Sciences) was distributed over the whole membrane. Membranes were then imaged using either a myECL™ Imager (Thermo Fisher Scientific) or Amersham Hyperfilm ECL (GE Healthcare Life Sciences) with exposure for up to 100 minutes.

Table 2. 1 List of Antibodies

A list of antibodies used in each assay. Antibodies are listed with their specificity (antigen), clonality, their target and host species, their conjugate (if any), the assays they have been used in, the distributor and product code. Table abbreviations: HC = heavy chain; MBL = mannose binding lectin; HRP = horseradish peroxidase; fluorescein isothiocyanate = FITC; poly = polyclonal; mono = monoclonal; AP = alkaline phosphatase 1 = ELISA; 2 = western blot; 3 = FPLC; 4 = complement pathway inhibition; 5 = haemolytic assay; 6 = flow cytometry; 7 = surface plasmon resonance, 8 = rabbit immunisation.

Antigen	Subclass	Clonality	Target Species	Host Species	Conjugate	Assay	Distributor	Product Code
IgG1 HC	IgG	Mono	Human	Rabbit	N/A	1, 2	Stratech	3831-1-EPI
IgG2 HC	IgG	Mono	Human	Rabbit	N/A	1, 2	Stratech	3410-1-EPI
IgG3 HC	IgG	Mono	Human	Rabbit	N/A	1, 2	Stratech	3338-1-EPI
IgG4 HC	IgG	Mono	Human	Rabbit	N/A	1, 2	Stratech	3479-1-EPI
IgG	Whole IgG	Poly	Human	Donkey	HRP	1, 2	Stratech	709-035-149-JIR
IgM Fc5µ	Whole IgG	Poly	Human	Donkey	HRP	1, 2	Stratech	709-005-073-JIR
IgG2 Fc	IgG1	Mono	Human	Mouse	N/A	3	Stratech	6002P-HRL
IgG	Whole IgG	Poly	Mouse	Goat	HRP	1, 2	Stratech	115-035-062-JIR
C1q	Whole IgG	Poly	Human	Sheep	HRP	1, 2	Bio-Rad	2221-5004P
C3	Whole IgG	Poly	Human	Goat	N/A	1, 2	Thermo Fisher	PA1-29715
IgG	Whole IgG	Poly	Goat	Rabbit	HRP	1, 2	Thermo Fisher	31402
MBL	IgG2A	Mono	Human	Mouse	N/A	1, 4	R&D Systems	MAB23071
IgG	Whole IgG	Poly	Goat	Rabbit	HRP	1, 2	Stratech	305-035-045-JIR
C1q	IgG1k	Mono	Human	Mouse	N/A	1, 4, 5	Pathway Diagnostics	A201
IgG4 HC	IgG1	Mono	Human	Mouse	N/A	1, 2	Life Technologies	A-10651
IgG1 HC	IgG1	Mono	Human	Mouse	N/A	1, 2	Life Technologies	A-10630
C3/C3c	Whole IgG	Poly	Human	Sheep	HRP	1, 2	antibodies-online	ABIN293935
IgG2	IgG1	Mono	Human	Mouse	N/A	1, 2	Life Technologies	MA1-83241
IgG3	IgG1	Mono	Human	Mouse	N/A	1, 2	Life Technologies	MA1-83242
IgM	Whole IgG	Poly	Human	Goat	HRP	1, 2	Life Technologies	A18841
IgG	Whole IgG	Poly	Mouse	Goat	HRP	1, 2	Life Technologies	31446
IgG	Whole IgG	Poly	Mouse	Donkey	HRP	1, 2	Life Technologies	A16017
RBC Stroma	Whole IgG	Poly	Sheep	Rabbit	N/A	5	Sigma-Aldrich	S1389
IgG/IgM	Whole IgG	Poly	Human	Goat	FITC	6	Jackson ImmunoResearch	109-095-003
C3c	Whole IgG	Poly	Human	Rabbit	FITC	6	Abcam	ab4212
C3c	Whole IgG	Poly	Rabbit	Goat	FITC	6	Acris	AP31546FC-N
IgG/IgM	Whole IgG	Poly	Human	Goat	HRP	1, 2	Jackson ImmunoResearch	109-035-127
C5b-9	Unknown	Unknown	Human	Unknown	AP	1	Euro Diagnostica	COMPL 300
IgG	Unknown	Poly	Human	Unknown	HRP	1	Thermo Fisher	991000
IgM	Unknown	Mono	Human	Unknown	HRP	1	eBioscience	88-50620-22
Factor H	IgG1	Mono	Human	Mouse	N/A	2, 3	In-house (OX-24)	N/A
Factor H	IgG1	Mono	Human	Mouse	N/A	2, 3	In-house (MBI-7)	N/A
Factor B	IgG2b	Mono	Human	Mouse	N/A	1, 4	In-house	Gifted from Dr Córdoba
N/A	IgM	Poly	N/A	Human	N/A	1, 6, 7	In-house	N/A
N/A	IgG1	Poly	N/A	Human	N/A	1, 6, 7	In-house	N/A
N/A	IgG2	Poly	N/A	Human	N/A	1, 6, 7	In-house	N/A
N/A	IgG3	Poly	N/A	Human	N/A	1, 6, 7	In-house	N/A
N/A	IgG4	Poly	N/A	Human	N/A	1, 6, 7	In-house	N/A
MenACWY	IgM	Poly	N/A	Human	N/A	1, 6, 7	In-house	N/A
MenACWY	IgG2	Poly	N/A	Human	N/A	1, 6, 7	In-house	N/A
MenACWY	IgG1	Poly	N/A	Human	N/A	1, 6, 7	In-house	N/A
MenACWY	IgG/IgM	Poly	N/A	Human	N/A	1, 6, 7	In-house	N/A
N/A	IgG	Poly	N/A	Rabbit	N/A	3	In-house	N/A
N/A	Whole IgG	Poly	N/A	Human	N/A	8	In-house	N/A

2.5 Enzyme-Linked Immunosorbent Assay (ELISA)

Unless otherwise stated, flat-bottomed 96 well-plates (Microplate Immuno MaxiSorp; Fisher Scientific) were coated with 90µL/well ligand diluted in 30mM Na_2CO_3 (Sigma), 70mM $NaHCO_3$ (Sigma) pH9.6 overnight at 4°C. Plates were washed between incubations three times with 180µL/well PBS, 0.1% (v/v) Tween20 using a plate washer (Thermo Fisher). Plates were blocked for at least one hour at ambient temperature with 180µL/well PBS, 0.1% (v/v) Tween20, 5% (w/v) bovine serum albumin (BSA, Sigma; blocking buffer). All samples were run in duplicate. Plates were developed with 90µL/well SIGMAFAST™ o-Phenylenediamine dihydrochloride (OPD; Sigma-Aldrich, Inc) for 5 to 20 minutes. The reaction was stopped with 45µL/well 10% H_2SO_4. Plates were read using an Infinite® F50 plate reader (Tecan, Reading, UK) at a wavelength of 492nm (A492nm). Antibodies used in each ELISA described below are detailed in ***Table 2.1***.

2.5.1 Competitive ELISA

2.5.1.1 Functional Affinity of Anti-MenACWY Antibodies to MenACWY Antigen

The functional affinity (molar; M) of human IgG1, IgG2, IgG3, IgG4 and IgM antibody present in the plasma of 14 adult individuals to TT-conjugated meningococcal polysaccharides from serogroups A, C, W and Y (MenACWY-TT) was measured by competitive ELISA. The plasma was taken at least one month post vaccination with a quadrivalent plain meningococcal polysaccharide (from serogroups A, C, W-135 and Y) vaccine (Mencevax™). Two ELISA plates were coated with 1µg/mL MenACWY-TT and blocked. Plasma samples, diluted in blocking buffer to a factor calculated as described in ***Section 2.5.1.2***, were each mixed with a titration of MenACWY-TT ($1x10^{-7}$ to $4x10^{-13}$M) and left to stand for one hour at ambient. A plasma only control was also included in the assay for each sample (no MenACWY-TT). A blocking buffer only negative control (no plasma) was also included in each assay. Each plasma:MenACWY-TT mixture was added to one plate at 90µL/well in triplicate for 15 minutes. Each

plasma:MenACWY-TT mixture was then aspirated from the first plate and transferred to the second plate at a volume of 90µL/well in duplicate for a further 15 minutes. Both plates were washed and bound antibody was detected with the addition of 90µL/well of either mouse anti-human IgG1 (1µg/mL; Stratech Scientific Limited, Suffolk, UK), mouse anti-human IgG2 (1µg/mL; Stratech), mouse anti-human IgG3 (1µg/mL; Stratech), mouse anti-human IgG4 (1µg/mL; Stratech) or donkey anti-human IgM-HRP (diluted 1/1000; v/v; Stratech) diluted in blocking buffer to both plates for one hour at ambient. Plates were washed and 90µL/well goat anti-mouse IgG-HRP, diluted 1/1000 (v/v) in blocking buffer, was added to each plate (apart from anti-IgM competitive ELISAs) for one hour at ambient. Plates were washed and developed.

The percentage inhibition of antibody binding to plates at each concentration of MenACWY-TT was calculated as:$1-\frac{(Plasma{:}MenACWYTT\ Sample\ A492)-(Blank\ A492)}{(Plasma\ with\ no\ MenACWYTT\ A492)-(Blank\ A492)}\times 100$. The concentration (M) of MenACWY inhibiting 50% antibody binding (functional affinity) was interpolated by plotting percentage inhibition of antibody binding versus concentration of MenACWY-TT. Significant differences between the functional affinities of anti-MenACWY-TT antibody subclasses were calculated by t-test analysis. Absorbance values from first and second plates for each plasma:MenACWY-TT mixtures were compared by linear regression (Prism).

2.5.1.2 Sample Dilution for Competitive ELISA

Plates were coated with 1µg/mL MenACWY-TT and blocked. Plasma samples were serially diluted 1/10-10,000 (v/v) in blocking buffer across the plate at a volume of 90µL/well in duplicate for 15 minutes at ambient. Plates were washed and 90µL/well of either mouse anti-human IgG1 (1µg/mL; Stratech), mouse anti-human IgG2 (1µg/mL; Stratech), mouse anti-human IgG3 (1µg/mL; Stratech), mouse anti-human IgG4 (1µg/mL; Stratech) or donkey anti-human IgM-HRP (diluted 1/1000; v/v; Stratech), diluted in blocking buffer, was added to plates and

incubated for one hour at ambient. Plates were washed and 90µL/well goat anti-mouse IgG-HRP, diluted 1/1000 (v/v) in blocking buffer, was added to each plate (excluding wells previously incubated with the anti-IgM-HRP antibody) and incubated for one hour at ambient. Plates were then washed and developed. The dilution factor of each antibody subclass in each plasma sample achieving an absorbance of 1.0 at 492nm was calculated with Prism by plotting absorbance against plasma dilution.

2.5.1.3 Functional Affinity of Purified Versus Plasma Anti-MenACWY IgG1 Antibody with MenACWY Antigen

The functional affinities of IgG1 antibody present in plasma and purified IgG1 antibody (from the same plasma sample) to TT-conjugated meningococcal polysaccharides were compared by competitive ELISA. IgG1 antibody was purified from a pool of human plasma (n=14 individuals), taken from adult individuals vaccinated with plain MenACWY, as described in ***Section 2.2.4***. The plasma was taken at least one month post vaccination with a quadrivalent plain meningococcal polysaccharide (from serogroups A, C, W-135 and Y) vaccine (Mencevax™). The competitive ELISA was performed with both samples as described in ***Section 2.5.1.1***.

2.5.2 Human and Rabbit C1q Binding ELISA

ELISA wells were coated with 30µg/mL of either non-specific human IgG1, IgG2, IgG3, IgG4, IgM, IgG, anti-MenACWY or non-specific rabbit IgG antibody, or left blank. Once blocked, 90µL/well 4% human serum diluted in blocking buffer, 4% rabbit serum diluted in blocking buffer or blocking buffer only was added to each antibody coating and incubated for two hours at ambient. Bound human and rabbit C1q was detected with 90µL/well goat anti-human C1q-HRP (Bio Rad), diluted 1/400 (v/v) in blocking buffer. After a one-hour incubation at ambient, plates were washed and developed.

2.5.3 Human and Rabbit Complement Activation by Immunoglobulins

ELISA wells were coated with 90µg/well 15µg/mL of either non-specific human IgG1, IgG2, IgG3, IgG4, IgM, IgG, anti-MenACWY or non-specific rabbit IgG antibody preparations, or left blank. Once blocked, 90µL/well 0.5% human serum diluted in complement fixation diluent (CFD), 10% rabbit serum diluted in CFD, or blocking buffer was added for one hour at ambient. Deposited human and rabbit C3 was detected with 90µL/well goat anti-human C3-HRP (Thermo Fisher), diluted 1/500 (v/v) in blocking buffer. After a one-hour incubation at ambient, plates were washed and developed.

2.5.4 Anti-Meningococcal Polysaccharide Complement Deposition Assay

ELISA wells were coated with 20µg/mL MenACWY-TT and blocked. A serial dilution of anti-MenACWY IgG1, IgG2 and IgM antibodies (40-0.04µg/mL) was added at 90µL/well and incubated for one hour at ambient. The anti-MenACWY IgG1, IgG2 and IgM antibodies were isolated from pooled plasma of 14 adult individuals previously vaccinated with a quadrivalent plain meningococcal polysaccharide (from serogroups A, C, W-135 and Y) vaccine (Mencevax™).

To each plate, 90µL/well 0.5% anti-MenACWY antibody-depleted human serum (diluted in CFD and prepared as described in ***Section 2.5.4.1***) or 10% rabbit serum (diluted in CFD) was added to each antibody titration and incubate at ambient temperature for 30 minutes. Bound human and rabbit C3 was detected with 90µL/well goat anti-human C3-HRP (Thermo Fisher), diluted 1/500 (v/v) in blocking buffer. After a one-hour incubation at ambient, plates were washed and developed.

2.5.4.1 Anti-MenACWY-TT Antibody-Depleted Human Serum

Endogenous reactivity to MenACWY-TT in the human serum used in the anti-meningococcal polysaccharide complement deposition assay (***Section 2.5.4***) was removed by depletion of anti-MenACWY-TT antibody. This was achieved with multiple passages of serum over a 5mL MenACWY-TT HiTrap NHS-Activated HP column.

To ensure no loss of complement activity had occurred during the depletion of anti-MenACWY-TT antibody, the haemolytic activity of the anti-MenACWY antibody-depleted human serum was compared to that of normal human serum as described in ***Section 2.8.1***.

To ensure endogenous reactivity to MenACWY-TT had been removed from human serum, C3 deposition and IgG binding, following incubation with MenACWY-TT was measured by ELISA and compared with normal human serum (C3 deposition and IgG binding) and rabbit serum (C3 deposition only). ELISA plates were coated with 20µg/mL MenACWY-TT and blocked. A serial dilution series of the anti-MenACWY antibody-depleted human serum, normal human serum and rabbit serum (20-0.3% diluted in CFD) was added at 90µL/well for 30 minutes at ambient. C3 deposition was detection with 90µL/well goat anti-human C3-HRP (Thermo Fisher), diluted 1/500 (v/v) in blocking buffer and bound IgG antibody was detected with 90µL/well anti-human IgG-HRP (Jackson ImmunoResearch), diluted 1/1000 (v/v) in blocking buffer. After a one-

hour incubation at ambient, plates were washed and developed. IgG binding and C3 deposition achieved with each serum was compared by two-way ANOVA.

2.5.5 WeissLab® Complement System Screen ELISA kit

Blockade of classical and alternative pathways of complement in human serum, incubated with various complement pathway inhibitors, was assessed using a WeissLab® Complement System Screen ELISA kit (COMPL 300; Euro Diagnostica, Malmö, Sweden) as described by Seelen et al., (2005).

Plates were pre-coated with either human IgM (classical pathway assay) or LPS (alternative pathway assay) and pre-blocked with PBS 1% (w/v) BSA. Human serum from three individuals was diluted 1/101 (v/v) in classical pathway diluent (CPD) or 1/18 (v/v) in alternative pathway diluent (APD) with or without 0.01M EDTA, 15nM anti-C1q (Pathway Diagnostics), 250nM anti-FB, 250nM anti-MBL (R&D Systems) and 1.6µM human FH and left at ambient temperature for 30 minutes. It is understood that diluent CP consists of veronal buffered saline (VBS) 5mM $MgCl_2$, 0.05% (v/v) Tween20, and 0.1% (w/v) gelatine, pH 7.5 and diluent AP consists of VBS 10mM EGTA, 5mM $MgCl_2$, 0.05% (v/v) Tween20, and 0.1% (w/v) gelatine, pH 7.5. The positive control (lyophilised human serum) was reconstituted with distilled water and left for 5 minutes at ambient temperature before diluting in either CPD or APD as before. The negative control (heat-inactivate human serum) was diluted in either CPD or APD as before.

Positive controls, negative controls, serum samples and CPD/APD (assay blanks) were added to the corresponding wells at 100µL/well and incubated for 65 minutes at 37°C. After incubation, wells were aspirated and washed with 300µL/well 1x wash buffer three times. After the final wash, 100µL/well anti-human C5b-9 alkaline phosphatase-conjugated detection antibody was added to each well and incubated at ambient temperature for 30 minutes. Wells were then aspirated and washed three times as before. The substrate solution was added to the

plate at 100µL/well and incubated for 30 minutes as ambient. To stop the reaction, 100µL/well 5mM EDTA was added. Plates were then read at a wavelength of 405nm (A405). The absorbance achieved with human serum in the presence of each inhibitor was compared to the absorbance achieved with human serum alone by t-test statistical analysis. The percentage inhibition of classical and alternative pathway activation achieved with each inhibitor was calculated as: absorbance achieved with serum sample plus the inhibitor over the absorbance achieved with serum alone.

2.5.6 Lectin Pathway Inhibition ELISA

The concentration of an anti-MBL antibody (R&D Systems) required to efficiently block complement activation via the lectin pathway in human serum was measured by ELISA. Firstly, ELISA plates were coated 200µg/mL mannan (from *Saccharomyces cerevisiae*; Sigma-Aldrich) and blocked. A serial dilution of human serum (10-0.01% diluted in CFD) was added at 90µL/well for 30 minutes at ambient. C3 deposition was detected with 90µL/well goat anti-human C3-HRP (Thermo Fisher), diluted 1/500 (v/v) in blocking buffer. After a one-hour incubation at ambient, plates were washed and developed. The concentration of human serum resulting in 80% maximum absorbance was interpolated by fitting a non-linear regression curve to the data.

A titration series of the anti-MBL antibody was incubated with the selected concentration of human serum resulting in 80% maximum absorbance for 30 minutes. Serum:anti-MBL mixtures were then added to a plate coated with mannan and subsequent C3 deposition was detected as previously described.

2.6 Anti-MenACWY Antibody Subclass ELISA

A series of ELISAs were developed to measure The concentration (µg/mL) or proportion (% of total antibody) of meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific IgG1, IgG2 and IgM antibody was measured in the serum of individuals at least one month or four months post-vaccination with either a plain (Mencevax™) or a TT-conjugated (Nimenrix™) quadrivalent meningococcal polysaccharide (serogroups A, C, W-135 and Y) vaccine. Sample were taken as part of two clinical trials (identifiers: NCT00427908 and NCT00718666) as described by Vesikaria et al., (2012) and Klein et al., (2013) as described in ***Section 2.6.3***.

2.6.1 Anti-MenACWY Antibody Standard Isolation

The anti-MenACWY antibody standard was isolated from the plasma of 14 adult individuals (40mL per person) as described in ***Section 2.2.3***. The plasma was taken at least one month post vaccination with a quadrivalent plain meningococcal polysaccharide (from serogroups A, C, W-135 and Y) vaccine (Mencevax™). Roughly 15.3mg anti-MenACWY antibody was isolated in total.

2.6.1.1 Anti-MenACWY Antibody Standard Reactivity ELISA

To ensure the anti-MenACWY antibody standard was specific to MenACWY only and devoid of anti-TT antibody, binding to both MenACWY-TT and TT was checked by ELISA. Plates were coated with either 5µg/mL TT or 5µg/mL MenACWY-TT and blocked. A titration of the anti-MenACWY antibody standard (2.5-0.01µg/mL) was added to each coating at 90µL/well for one hour at ambient. Two wells for each coating were incubated with 90µL/well blocking buffer as the assay blanks. After washing three times, TT and MenACWY-TT bound IgG/IgM antibody was detected with 90µL/well goat anti-human IgG/IgM-HRP (Jackson ImmunoResearch Inc. PA, USA),

diluted 1/1000 (v/v) in blocking buffer. After a one-hour incubation at ambient, plates were washed and developed.

2.6.1.2 Anti-MenACWY Antibody Standard IgM Concentration ELISA

The concentration of IgM present in the anti-MenACWY standard was assessed using a Ready-SET-Go! ® Human IgM ELISA kit (eBioscience, Ltd, Cheshire, UK). A Corning Costar 9018 ELISA plate was coated overnight at 4°C with 100µL/well with anti-human IgM capture antibody diluted 1/250 (v/v) with PBS. Wells were aspirated and washed twice with 400µL/well PBS 0.05% (v/v) Tween20. Plates were then blocked for two hours at ambient temperature with 1/10 (v/v) dilution of assay buffer A concentrate (20x PBS with 1% Tween 20 and 10% BSA). Plates were aspirated and washed twice as before.

The recombinant IgM antibody standard provided (lyophilized) was reconstituted with distilled water to a concentration of 2µg/mL and allowed to stand at ambient temperature for 20 minutes. The antibody standard was then serially diluted 1 in 2 across the plate in duplicate, five times with 1/20 (v/v) assay buffer A concentrate to a final volume of 100µL/well (1000 to 15.63ng/mL). The anti-MenACWY antibody standard was diluted 1/800 (v/v), 1/1600 (v/v) and 1/3200 (v/v) in 1/20 (v/v) assay buffer A concentrate and added to the plate at 100µL/well. An in-house purified IgM antibody (isolated as described in ***Section 2.2.2***) diluted to 700ng/mL and 200ng/mL was added to each plate at 100µL/well as the assay control. Samples were incubated for two hours at ambient. Wells were then aspirated and washed as before four times. An anti-human IgM-HRP conjugated antibody, diluted 1/250 (v/v) with 1/20 (v/v) assay buffer A concentrate, was added to each well at 100µL/well for one hour at ambient. Plates were aspirated and washed four times as before.

Tetramethylbenzidine (TMB) substrate solution was added to each well at 100µL/well for 15 minutes followed by 100µL/well 2N H_2SO_4. Plates were read immediately at a wavelength of 450nm with absorbance values at 570nm subtracted. Graph Pad Prism software was used to interpolate the concentration of IgM present in the anti-MenACWY antibody standard using the recombinant IgM antibody dilution series as the standard curve.

2.6.1.3 Anti-MenACWY Antibody Standard IgG Subclass Concentration ELISA

The concentration of IgG1, IgG2, IgG3 and IgG4 present in the anti-MenACWY antibody standard was assessed using a IgG Subclass ELISA Kit (Thermo Fisher Scientific). To a pre-coated plate 50µL/well monoclonal anti-human IgG1/IgG2/IgG3 or IgG4 antibody was added to each well apart from blank wells, to which 50µL/well assay diluent buffer and 50µL/well anti-MenACWY antibody standard (diluted 1/40 (v/v)) was added. Plates were pre-coated in an IgG antibody specific for the anti-human IgG1/IgG2/IgG3 or IgG4 capture antibodies. Apart from blank wells, 50µL/well anti-MenACWY antibody diluted 1/40, 1/80, 1/160, 1/320 and 1/640 (v/v) was added to each IgG subclass capture antibody. The lyophilized human IgG subclass assay standard was reconstituted with the assay diluent buffer to a concentration of 13.72µg/mL IgG1, 5.32µg/mL IgG2, 1.34µg/mL IgG3 and 0.76µg/mL IgG4 and allowed to stand at ambient temperature for 10 minutes. The antibody standard was then serially diluted 1 in 2, five times (neat to 1/32(v/v)) with diluent buffer and 50µL/well of each dilution was added to each IgG subclass capture antibody. Finally, the human serum control (lyophilized) was reconstituted with 1mL diluent buffer to a concentration of 1.97-2.59µg/mL IgG1, 0.78-1.40µg/mL IgG2, 0.19-0.30µg/mL IgG3 and 0.16-0.23µg/mL IgG4 and left to stand at ambient temperature for 10 minutes. The human serum control was added to each IgG subclass capture antibody at 50µL/well. The plate was then incubated for 30 minutes at ambient. Wells were aspirated and washed by adding 400µL/well and soaking for 30 seconds three times.

Anti-human IgG-HRP conjugated detection antibody was diluted 1/50 (v/v) in diluent buffer and added to each well at 100µL/well for 30 minutes at ambient. Plates were then washed three times as before and 100µL/well TMB solution was added for 10 minutes at ambient. After 10 minutes of developing, 100µL/well *stop solution* was added and plates read immediately at a wavelength of 450nm with absorbance values at 650nm subtracted. Graph Pad Prism software was used to interpolate the concentration of IgG1, IgG2, IgG3 and IgG4 present in the anti-MenACWY antibody standard using the four human IgG subclass antibody dilution series as the standard curves.

2.6.2 Anti-MenACWY Antibody Subclass ELISA Protocol

ELISA plates were coated with 4µg/mL meningococcal polysaccharides from serogroups A, C, W or Y plus 4µg/mL methylated human serum albumin (mHSA; National Institute for Biological Standards and Control, Hertfordshire, UK) and blocked. mHSA is used to attach meningococcal polysaccharides to ELISA plates (Arakere and Frasch, 1991). Plates were washed using a SkanWasher 300 Microplate Washer (Molecular Devices (UK) Limited, Berkshire, UK). Heat-inactive serum samples (cohorts detailed in ***Section 2.6.3***), diluted 1/5 (v/v) to 1/160 (v/v) in blocking buffer, were added to the plate at 90µL/well for one hour at 37°C in duplicate. The purified anti-MenACWY antibody standard was serially diluted 1 in 2 with blocking buffer from 10µg/mL to 0.02µg/mL and added to each plate at 90µL/well for one hour at 37°C. Two controls, each created by pooling the plasma of 10 vaccinated individuals, were included in each assay.

Plates were washed three times and bound IgG1, IgG2 and IgM antibody was detected with 90µL/well mouse anti-human IgG1 antibody diluted 1/1000 (v/v) in blocking buffer, mouse anti-human IgG2 antibody diluted 1/1000 (v/v) in blocking buffer or goat anti-human IgM HRP conjugated antibody diluted 1/1000 (v/v) in blocking buffer (all anti-Ig were from Life Technologies). After one hour incubation at 37°C, 90µL/well goat anti-mouse IgG-HRP-

conjugated antibody (Life Technologies) diluted 1/1000 (v/v) in blocking buffer was added to each plate (except those to which goat anti-human IgM HRP conjugated antibody was added) and incubated for one hour at 37°C.

Plates were washed, developed and read using a VersaMax ELISA Microplate Reader (Molecular Devices) at a wavelength of 492nm (A492nm). Graph Pad Prism software was used to interpolate the concentration of anti-MenA/C/W/Y IgG1, IgG2 and IgM present in each sample.

2.6.2.1 Intra-Assay and Inter-Assay Coefficient of Variation

The intra-assay coefficients of variation of each anti-MenACWY IgG1, IgG2 and IgM assay was calculated by running two samples (each created by pooling the plasma of 10 vaccinated individuals) through each assay, as described in ***Section 2.6.2***, seven times. Each of the seven repeats were run in the same assay and antibody concentration interpolated from one antibody standard. The coefficient of variation was calculated as the standard deviation divided by mean titre and expressed as the percentage of variation (CV %).

The inter-assay coefficients of variation of each anti-MenACWY IgG1, IgG2 and IgM assay was calculated by running two samples (each created by pooling the plasma of 10 vaccinated individuals) through each assay, as described in ***Section 2.6.2***, five times. Each of the five repeats were run in separate assays and antibody concentration was interpolated from separate antibody standards. The coefficient of variation was calculated as the standard deviation divided by mean titre and expressed as the percentage of variation (CV %).

2.6.2.2 Assay Lower Limit of Detection

The lower limits of detection for each anti-MenACWY IgG1, IgG2 and IgM assay was interpolated from the average absorbance achieved by a negative sample run in five sequential assays plus three standard deviations.

2.6.3 Assay Cohorts and Vaccine Details

Plasma samples were collected during two clinical trials assessing the immunogenicity of a tetanus toxoid conjugated quadrivalent meningococcal vaccine (Nimenrix™; GSK134612; GlaxoSmithKline Biologicals SA) in infants and children (Klein et al., 2013; Vesikari et al., 2012). The first clinical trial, comparing the response to a single dose Nimenrix™ and a plain quadrivalent meningococcal vaccine (Mencevax™, GlaxoSmithKline Biologicals SA) one month post vaccination in children 2-10 years of age, is registered at ClinicalTrials.gov (identifier: NCT00427908, 2012). Twenty-two samples from subjects vaccinated with Mencevax™ and twenty-eight samples from subjects vaccinated with Nimenrix™ were run through each of the anti-MenA/C/W/Y antibody subclass ELISAs. The second clinical trial, comparing the response to one or two doses of Nimenrix™ four months post final vaccination in babies 9-12 months of age, is registered at ClinicalTrials.gov (identifier: NCT00718666, 2013). Twenty-three samples from subjects vaccinated with one dose Nimenrix™ and twenty-six samples from subjects vaccinated with two doses Nimenrix™ were run through each of the each anti-MenACWY IgG1, IgG2 and IgM assay

Each dose of Nimenrix™ comprises 5µg *Neisseria meningitidis* group A polysaccharide, 5µg *Neisseria meningitidis* group C polysaccharide, 5µg *Neisseria meningitidis* group W-135 polysaccharide, 5µg *Neisseria meningitidis* group Y polysaccharide and 44µg tetanus toxoid conjugate. Each dose of Mencevax™ contains 50µg *Neisseria meningitidis* group A polysaccharide, 50µg *Neisseria meningitidis* group C polysaccharide, 50µg *Neisseria meningitidis*

group W-135 polysaccharide and 50µg *Neisseria meningitidis* group Y polysaccharide. Both vaccines were administered by intramuscular injection.

SBA titres of serum samples towards *Neisseria meningitidis* serogroups A (strain 3125), C (strain C11), W-135 (strain MP01240070) and Y (strain 1975) were assessed during clinical trials NCT00427908 and NCT00718666. SBAs were performed at either GlaxoSmithKline Vaccines, Wavre, Belgium by Vesikari et al., (2012) or GlaxoSmithKline Vaccines, Rixensart, Belgium by Klein et al., (2013). The source of human complement used in the hSBA was a non-depleted human serum that was specifically screened to determine absence of antibodies to *Neisseria meningitidis* and absence of intrinsic toxicity.

2.6.4 Anti-MenACWY Antibody Subclass Composition – Pilot Study

Anti-MenACWY antibody was isolated individually from the plasma of each of eleven adult individuals as described in ***Section 2.2.3***. The plasma was taken at least one month post vaccination with a quadrivalent plain meningococcal polysaccharide (from serogroups A, C, W-135 and Y) vaccine (Mencevax™). Anti-MenACWY antibody from each individual was run through both the IgG Subclass ELISA Kit and Ready-SET-Go! ® Human IgM ELISA Kit as described in ***Section 2.6.1.2*** and ***Section 2.6.1.3***, respectively. The percentage of IgG1, IgG2, IgG3, IgG4 and IgM anti-MenACWY antibody present in each sample was calculated as: (antibody subclass concentration (µg/mL) over total antibody concentration (µg/mL)) x100.

2.7 Surface Plasmon Resonance (SPR)

The interaction of human IgG1, IgG2, IgG3, IgG4 and IgM with human and rabbit C1q was measured by SPR as described by Patel et al., (2015). SPR assays were run on a Biacore™ T200 System at a flow rate of 30µL/minute with 0.01M HEPES pH 7.4, 0.15M NaCl, 3mM EDTA, 0.005% v/v Surfactant P20 (HBS) as the running buffer. All SPR reagents were from GE Healthcare.

Recombinant Protein L (Life Technologies Ltd) was amine coupled to a CM5 Biacore™ sensor chip using an Amine Coupling Kit following supplier's instructions. The chip surface was activated with a 400 second injection of 11.5mg/mL N-Hydroxysuccinimide and 75mg/mL 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride mixed 1/1 (v/v) at a flow rate of 10µL/minute. Protein L was diluted to 0.1mg/mL in 10mM sodium acetate pH4.0 and injected for 480 seconds over each flow cell at a flow rate of 10µL/minute. Residual active groups on flow cells were deactivated with a 400 second injection of 1M ethanolamine hydrochloride-NaOH pH8.5 at a flow rate of 10µL/minute. The flow cell surface was then washed with a 60 second injection of 50mM NaOH. Flow cell 2 was used to capture each antibody subclass and flow cell 1 was used as the reference surface. Non-specific human IgG1, IgG2, IgG3, IgG4 and IgM antibodies, prepared from human serum as described in ***Section 2.2.4***., were captured to flow cell 2 to levels between 40-300 response units (RU). Rabbit or human C1q, isolated as described in ***Section 2.2.5*** and ***Section 2.5.6***, were diluted in HBS to 600-3.125nM and injected over flow cells 1 and 2 for 20-40 seconds. Between cycles, chip surfaces were regenerated with a 180 second injection of 10mM Glycine-HCl pH1.5 at a flow rate of 10µL/minute. The affinity of C1q for each antibody subclass was calculated using Biacore™ T200 Evaluation Software (version 2.0.1025; GE Healthcare) by steady state analysis. A graphical summary of the protocol used to assess the affinity of immunoglobulin binding of human and rabbit C1q is shown in ***Figure 2. 1***.

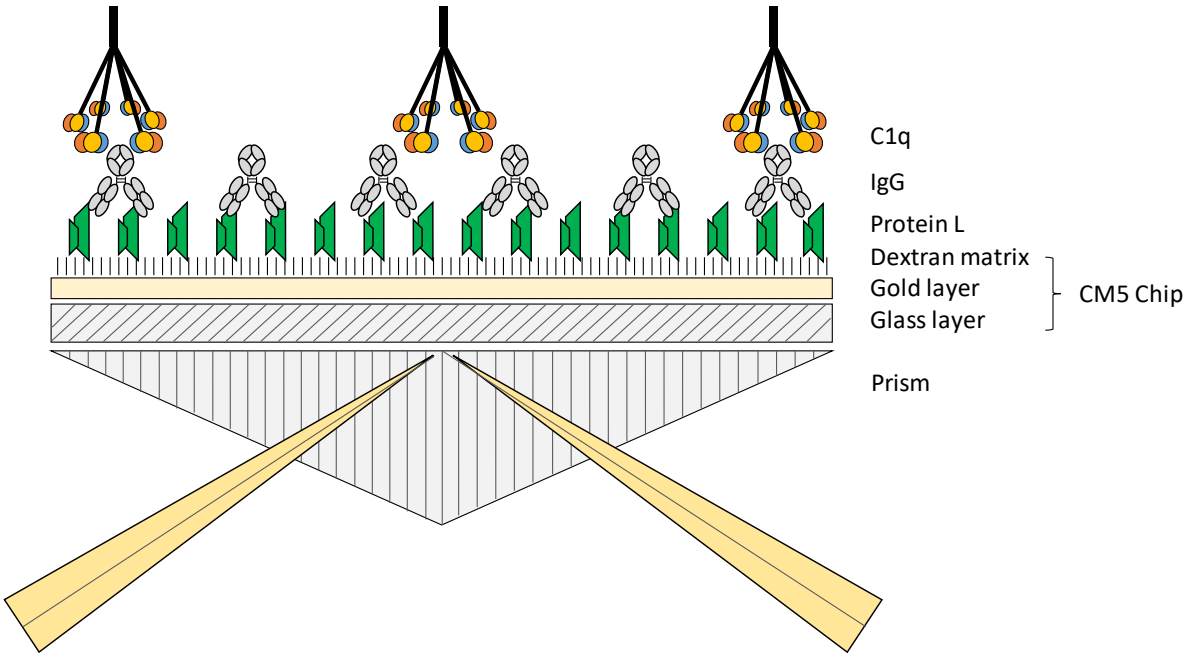


Figure 2. 1 Surface Plasmon Resonance (SPR) Assay Layout
The interaction of purified human IgG1, IgG2, IgG3 and IgG4 to purified human and rabbit C1q was assessed by surface plasmon resonance (SPR). Human or rabbit C1q was injected over antibody subclasses captured to a protein L coated CM5 Biacore™ sensor chip.

2.8 Haemolytic Assays

2.8.1 Classical Pathway Haemolytic Assay

Human or rabbit serum was serially diluted (20% starting concentration) across a U-bottomed plate by 2/3 (v/v) dilutions in CFD to a final volume of 50µL/well in duplicate. Antibody-sensitised sheep erythrocytes (EA; 2% suspension in CFD) prepared as described in **Section 2.8.1.1**., were added to each serum concentration (50µL/well) followed by 50µL/well CFD. Negative control comprising EA in CFD only (0% haemolysis) and 100% lysis control comprising EA in H_2O were included in each assay. Plates were sealed and incubated at 37°C for 30 minutes. After incubation, plates were spun at 1500RPM for 5 minutes at ambient temperature and 100µL/well supernatant was carefully aspirated and transferred to a flat bottomed plate without disturbing the pellet. Supernatant absorbance was read using a VersaMax ELISA Microplate Reader (Molecular Devices) at a wavelength of 405nm (A405). Percentage lysis at each serum concentration was calculated as: $\frac{(Serum\ A405)-(Negative\ Control\ A405)}{(Positive\ Control\ A405)-(Negative\ Control\ A405)} \times 100$. GraphPad Prism software was used to calculate the serum concentration resulting in 50% haemolysis by plotting log concentration of serum against percentage haemolysis and fitting with a non-linear regression equation.

2.8.1.1 Antibody sensitisation of Sheep Erythrocytes

Erythrocytes were pelleted by centrifugation from between 1-2mL of sheep blood in Alsever's solution (TCS Biosciences Ltd, Buckingham, UK) and the cell pellet was washed in 20mL CFD three times at ambient temperature by centrifugation at 2000RPM for 5 minutes. After the final wash, 400µL of the erythrocyte pellet was aspirated and re-suspended in 20mL CFD (2% suspension) pre-heated to 37°C. Rabbit anti-sheep erythrocyte antiserum (Sigma-Aldrich, 1/4000 (v/v in CFD)) was added to the erythrocyte suspension and incubated for 30 minutes at

37°C with occasional mixing. Excess antibody was removed by washing three times as before and the antibody-sensitised erythrocytes (EA) were re-suspended in 20mL CFD (2% EA suspension).

2.8.1.2 Classical Pathway Inhibition

The concentration of a blocking anti-C1q monoclonal antibody (Pathway Diagnostics) required to block classical pathway activation was assessed by classical pathway haemolytic assay. Human serum (3.5%) was incubated with different amounts of anti-C1q (40-0nM) for 30 minutes at ambient. Aliquots of each mixture were added to a U-bottomed plate in duplicate (50µL/well). EA (50µL; 2% suspension) were added to each well followed by 50µL/well CFD. The assay was continued as described in ***Section 2.8.1***.

2.8.2 Alternative Pathway Haemolytic Assay

Erythrocytes were pelleted by centrifugation from between 1-2mL of rabbit blood in Alsever's solution (TCS Biosciences Ltd) and the pellet washed in 20mL CFD 0.01M EGTA three times at ambient. After the final wash, 400µL of the pellet was aspirated and re-suspended in 20mL CFD containing 0.01M EGTA, creating a 2% suspension of rabbit erythrocytes (RbE).

Human serum was titrated (20% starting concentration) across a U-bottomed plate by 2/3 (v/v) dilutions in CFD 0.01M EGTA to a final volume of 50µL/well in duplicate. RbE (2% suspension) were added to each serum concentration of serum (50µL/well) followed by 50µL/well CFD 0.01M EGTA. Plates were sealed and incubated at 37°C for 30 minutes. After incubation, plates were spun at 1500RPM for 5 minutes at ambient temperature and 100µL/well was carefully aspirated and transferred to a flat-bottomed plate without disturbing the pellet.

Supernatant absorbance was read at a wavelength of 405nm (A405). Percentage lysis at each serum concentration was calculated as described in ***Section 2.8.1***.

2.8.2.1 Alternative Pathway Inhibition

The titre of a blocking anti-FB monoclonal antibody required to block alternative pathway activation was assessed by alternative pathway haemolytic assay. The anti-FB antibody was kindly provided by Dr. Santiago Rodríguez de Córdoba (Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, Madrid) which was generated by immunising FB-deficient mice with purified human FB. Human serum (3.5%; 0.01M EGTA) was incubated with different amounts of anti-FB (40-0nM) for 30 minutes at ambient. Each mixture was added to a U-bottomed plate in duplicate (50µL/well). RbE (2% suspension in CFD 0.01M EGTA) were added to each serum concentration (50µL/well) followed by 50µL/well CFD 0.01M EGTA. The assay was continued as described in ***Section 2.8.2***.

2.9 Serum Bactericidal Assay

Undiluted, heat-inactivated immune plasma samples or purified anti-MenACWY IgG1, IgG2 or IgM antibodies were doubling diluted (neat to 1 in 512 (v/v) in HBSS containing 0.5% BSA) across a U-bottomed plate to a final volume of 20µL/well. Live *Neisseria meningitidis* bacteria (serogroup W-135; strain 102/98; prepared as described in ***Section 2.9.1***; 10µL at $6x10^4$ cells/mL in HBSS 0.5% BSA) were added to each well, followed by IgG-depleted human serum or BRS (Cedarlane) at 10µL/well. IgG depleted human serum was prepared as described by Brookes et al., (2013). Plates were incubated for one hour at 37°C with shaking at 65RPM using an iEMS^{TM} shaking incubator. Controls included bacteria plus serum only, bacteria plus heat inactivated serum only, and bacteria plus test immune sample plus heat-inactivated serum. Bacteria (10µL

aliquots) taken before the incubation and after the one hour incubation under the stated conditions were plated out onto a blood agar plate (bioMerieux, MO, USA) using the *tilt* method (T0; 0% killing). The *tilt* method involves transferring 10µL of sample to the top of a blood agar plate, angled at roughly 45°, and allowing the sample to trickle down to the bottom of the plate. Plates were left upright for ten minutes before inverting and incubating overnight at 37°C. The number of colonies (colony forming units, CFU) was then counted for each sample.

2.9.1 Preparation of Neisseria meningitidis *for Serum Bactericidal Assay*

Neisseria meningitidis serogroup W-135 (strain 102/98) bacteria from frozen stocks were streaked on a blood agar plate and incubated overnight at 37°C. Frantz medium (11mM L-glutamic acid, 103mM NaCl, 17mM $Na_2HPO_4.7H_2O$, 23mM NH_4Cl, 1.2mM KCl, 0.1mM L-cysteine hydrochloride monohydrate, 2g/L dialysed yeast extract, 5mM $MgSO_4.7H_2O$, 28mM glucose, pH7.4) was then inoculated with individual clones to $2x10^8$ cells/mL (absorbance of 0.1 at 600nm) and grown for three hours at 37°C. Bacteria were diluted to $4x10^4$ cells/mL in HBSS 0.5% BSA before use in assays.

2.9.2 Complement Inhibition in SBAs

The contribution of each pathway of complement activation to killing in SBAs was assessed using pathway-specific inhibitors. For classical pathway inhibition, an anti-human C1q antibody (A201; Pathway Diagnostics) was added to IgG-depleted human serum to a concentration of 15nM. For lectin pathway inhibition, an anti-MBL monoclonal antibody (MAB23071; R&D Systems) was added to IgG-depleted human serum and BRS to a concentration of 250nM. For alternative pathway inhibition, an anti-FB antibody (kindly provided by Dr. Santiago Rodríguez de Córdoba and prepared as described in ***Section 2.8.2.1***) or human FH

(prepared in house as described in ***Section 2.2.7***) was added to IgG-depleted human serum and BRS to a concentration of 250nM and 1.94µM, respectively. All inhibitor doses were selected to give complete inhibition of the respective pathway in the assays described above. The number of CFUs was counted after repeating the SBA assay with or without each complement pathway inhibitor as described in ***Section 2.9***.

2.10 Flow Cytometry

Flow cytometric assays were performed using a CyAn flow cytometer (Beckman Coulter (UK) Ltd, High Wycombe, UK) with an automated 96-well plate sampler (Cytek Biosciences Inc, CA, USA.

2.10.1 Preparation of Neisseria meningitidis *Bacteria for Flow Cytometric Assays*

Neisseria meningitidis serogroup W-135 (strain 102/98) bacteria were grown for four hours at 37°C in 5% (v/v) CO^2 in 10mL Frantz medium. Bacteria were then washed by centrifugation at 3060g for 5 minutes at 4°C and re-suspension in 10mL PBS, three times. After the final spin, bacteria were killed by re-suspension in PBS containing 0.2% (w/v) sodium azide and 17µg/mL phenylmethylsulfonyl fluoride and incubation for 48 hours at 37°C with 5% CO_2. The killed bacteria were plated overnight on Columbia agar with 5% horse blood (bioMérieux, MO, USA) to confirm killing. Bacteria were washed as before in PBS and diluted to $2x10^9$ bacteria/mL.

2.10.1.1 Preparation of Neisseria meningitidis *Bacteria for Opsonophagocytic Assays*

Neisseria meningitidis serogroup W-135 (strain 102/98) was grown for four hours at 37°C in 5% (v/v) CO^2 in 10mL Frantz medium. Bacteria were then washed by centrifugation at 3060g for 5 minutes at 4°C and re-suspension in 10mL PBS three times. After the final spin, bacteria were re-suspended in PBS and stained with 10µg/mL 2',7'-*bis*-(2- carboxyethyl)-5-(and-6) carboxyfluorescein acetoxymethyl ester (BCECF-AM) (Life Technologies). The bacteria were then killed as described in ***Section 2.10.1***.

2.10.2 Immunoglobulin Deposition Assay

Undiluted, heat-inactivated immune serum samples or purified anti-MenACWY IgG1, IgG2 and IgM antibodies were added to a U-bottomed plate at 2µL/well. Killed *Neisseria meningitidis* serogroup W-135 (strain 102/98) bacteria, prepared as described in ***Section 2.10.1***, were added to each well at 198µL/well $2x10^9$ bacteria/mL and incubated at 25°C for 30 minutes with shaking at 900RPM (Thermo Fisher Scientific Inc). Plates were then centrifuged at 3060g for 5 minutes at 4°C. Supernatants were aspirated and pellets were re-suspended in 200µL/well 2% BSA PBS. Centrifugation and re-suspension was repeated a further two times to remove unbound antibody. After the final wash, the pellet was re-suspended in 200µL/well goat anti-human IgG/IgM FITC antibody (Jackson Immunochemicals) diluted 1/500 (v/v) in 2% BSA PBS and incubated at 4°C for 20 minutes. Stained cells were washed as before, re-suspended in 200µL/well 2% BSA PBS and analysed by flow cytometry on the day of the assay. The fluorescence of 10,000 bacteria/sample (elliptical gating over *Neisseria meningitidis* population) was analysed in the fluorescence channel. A horizontal gate was enforced above the fluorescence of bacteria only samples.

2.10.3 Complement Deposition Assay

Undiluted, heat-inactivated immune plasma samples or purified anti-MenACWY IgG1, IgG2 and IgM antibodies were added to a U-bottomed plate at 5µL/well. BRS or IgG-depleted human serum was added to each well at 5µL/well, apart from bacteria only control and bacteria and conjugate antibody-only control. A bacteria plus complement only control was also included as the negative control. Killed *Neisseria meningitidis* serogroup W-135 (strain 102/98) bacteria (prepared as described in ***Section 2.10.1***) were added to each well at 90µL/well at $2x10^9$ bacteria/mL and incubated at 25°C for 45 minutes with shaking at 900RPM. Bacteria were washed three times by centrifugation at 3060g for 5 minutes at 4°C, aspiration of supernatant and pellet re-suspension in 200µL/well. After the final wash, pellets were re-suspended in 200µL/well sheep anti-human C3c-FITC (Abcam, Cambridge, UK) or sheep anti-rabbit C3c-FITC (Acris, MD, USA) diluted 1/500 (v/v) in 2% BSA PBS at 4°C for 20 minutes. Bacteria were washed as before, re-suspended in 200µL/well HBSS and analysed by flow cytometry on the day of the assay. The median fluorescence (complement deposition) of 10,000 bacteria/sample (elliptical gating over *Neisseria meningitidis* population) between samples was compared by t-test statistical analysis.

2.10.4 Opsonophagocytic Assay

Opsonophagocytic assays were performed as described by Humphries et al., (Humphries et al., 2015). Undiluted, heat-inactivated immune plasma samples or purified anti-MenACWY IgG1, IgG2 and IgM antibodies were added to a U-bottomed plate at 5µL/well. Dulbecco's PBS (DPBS) with 0.5% (w/v) BSA, 5mM glucose, 0.9mM $CaCl_2.2H_2O$ and 0.5mM $MgSO_4.H_2O$ (DPBS-GACM) was added to each well at 15µL/well. BCECF-AM-labelled killed *Neisseria meningitidis* serogroup W-135 (strain 102/98) bacteria (prepared as described in ***Section 2.10.1.1***) were added at 20µL/well at $2x10^9$ bacteria/mL and incubated for 30 minutes at 37°C whilst shaking at

900RPM with a iEMS^{TM} shaking incubator. BRS diluted 1/10 (v/v) in DPBS-GACM or IgG-depleted human serum diluted 1/10 (v/v) in DPBS-GACM was added at 10µL/well and incubated for 15 minutes at 37°C whilst shaking at 900RPM. Differentiated human promyelocytic leukaemia (HL60) cells (prepared as described in ***Section 2.10.4.1***) were added at 40µL/well at 2.5x10^7 cells/mL and incubated for 30 minutes at 37°C whilst shaking at 900RPM (multiplicity of infection (MOI), 40). A HL60 cells and bacteria only control and a HL60, bacteria and complement only control was included in each assay. After incubation, plates were immediately placed on ice and 80µL/well ice-cold DPBS 0.01M EDTA to stop the reaction. Before analysing by flow cytometry, 50µL/well of trypan blue was added to each plate to quench the fluorescence of un-phagocytosed bacteria. The median fluorescence (phagocytosis of BCECF-stained *Neisseria meningitidis*) of 10,000 HL60 cells/sample (elliptical gating over HL60 population) was analysed in the fluorescence channel. A horizontal gate was enforced above the fluorescence of HL60 cells only samples.

2.10.4.1 Differentiation of Human Promyelocytic Leukemia (HL60) Cells

HL60 cells (CCL-240™; American Type Culture Collection, Manassas, VA, USA) were differentiated into granulocyte-like cells as described by Humphries et al., (Humphries et al., 2015). HL60 cells, at a density between 1x10^5 and 1x10^6 cells/mL were incubated in RPMI 1640 medium (without phenol red; Thermo Fisher) supplemented with 20% FBS (w/v; LabTech), 1% L-glutamine (w/v; Thermo Fisher) and 0.8% (v/v) N,N-dimethylformamide (Sigma) for five days at 37°C in 5% CO_2. Before each assay, differentiated HL60 cells were diluted in DPBS-GACM to a concentration of 2.5x10^7 cells/mL.

2.10.5 Complement Inhibition in Flow Cytometric Assays

The complement deposition and opsonophagocytic assays were run with human and rabbit serum in the presence or absence of the described pathway-specific complement inhibitors. IgG-depleted human serum and BRS was pre-incubated with each pathway-specific inhibitor as described in ***Section 2.9.2***. Complement deposition on the bacteria and phagocytosis of the bacteria, with or without each of the complement pathway inhibitors was measured as described in ***Section 2.10.3*** and ***Section 2.10.4***, respectively.

2.11 Data Analysis

Apart from the exceptions that will be described below, all figures were generated using GraphPad Prism version 6.0.7 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. Statistical analyses of data (such as t-tests, two-way ANOVAs, one-way ANOVAs, linear regressions and non-linear regressions, standard deviations, means, geometric means and 95% confidence intervals) was performed using GraphPad Prism version 6.0.7 for Windows. All t-tests performed were two-tailed and paired. In ELISAs using a standard of a known concentration or in SDS-PAGE gels using a molecular weight marker, unknown concentrations and molecular weights were interpolated by fitting a 4PL sigmoidal, non-linear regression standard curve using GraphPad Prism version 6.0.7 for Windows.

For SPR assays, sensograms and KD values (steady state analysis) were generated using Biacore™ T200 Evaluation Software (version 2.0.1025; GE Healthcare). Chromatographs were generated using UNICORN Control Software (GE Healthcare Life Sciences). Analysis of flow cytometry data was performed using FlowJo® Single Cell Analysis Software (version 10.1; FlowJo, LLC, Ashland, Oregon). SDS-PAGE gels stained with Coomassie Brilliant Blue and western blots were imaged using with a myECL™ Imager (Thermo Fisher Scientific, Massachusetts, USA).

Chapter Three – Humoral Immune Response to Vaccination with Meningococcal Polysaccharide Conjugate

3.1 Introduction

The immunogenicity of meningococcal polysaccharide vaccines is routinely assessed by SBA using either human serum or BRS as the source of complement. The induction of bactericidal antibody is an important factor in SBA titres and protection against invasive meningococcal disease (Goldschneider et al., 1969a; Goldschneider et al., 1969b). However, the relationship between SBA titres and the concentration of anti-polysaccharide antibody is unclear.

Studies assessing the relationship between the concentration of anti-polysaccharide IgG and rSBA and hSBA titres show significant variation between serogroups (Borrow et al., 2001b; Bårnes et al., 2011; Elias et al., 2013; Findlow et al., 2009; Granoff et al., 1998; Guirola et al., 2006; King et al., 1996; Lee et al., 2014; Lieberman et al., 1996; Maslanka et al., 1998; Mitchell et al., 1996; Sikkema et al., 2000). One study, analysing the association between IgG response to a plain meningococcal quadrivalent polysaccharide vaccine and rSBA titres, showed a strong correlation for anti-MenC IgG titres (R square = 0.67) followed by anti-MenW IgG titres (R square = 0.58), anti-MenA IgG titres (R square = 0.38) and a very weak correlation with anti-MenY IgG titres (R square = 0.09) (Elias et al., 2013). To our knowledge no study has assessed the correlation between the antibody response to a meningococcal quadrivalent polysaccharide vaccine and hSBA titres.

Higher affinity antibody responses to meningococcal vaccines are associated with higher SBA titres (Hetherington and Lepow, 1992; Schlesinger et al., 1992). For this reason, it is thought that lower affinity antibodies contribute little to SBA titres. There is a concern that the standard ELISA used to measure the concentration of anti-polysaccharide antibody in vaccinee sera does

not distinguish between high and low-affinity antibodies and that the inclusion of low affinity antibodies, which do not significantly add to SBA titres, to the total concentration of antibody response to vaccination may account for the poor correlation seen between SBA titres and the concentration of anti-polysaccharide IgG (Elias et al., 2013; Findlow et al., 2009; Granoff et al., 1998; King et al., 1996; Lieberman et al., 1996). To address this issue, Granoff et al., added the chaotropic agent ammonium thiocyanate to vaccinee sera allowing antibodies only of the highest affinity to bind to the meningococcal polysaccharide-coated ELISA plates (Ferreira and Katzin, 1995; Granoff et al., 1998; MacDonald et al., 1988; Pullen et al., 1986). By selectively measuring the concentration of high-affinity anti-polysaccharide IgG only, the correlation between SBA titres and concentration of anti-polysaccharide IgG did significantly improve. The R values increased from 0.45 in the standard ELISA to 0.85 in the modified ELISA.

The observation that the concentration of high-affinity anti-polysaccharide IgG correlates with SBA titres better than the concentration of total (high and low affinity) IgG may explain why the correlation between SBA titres and anti-polysaccharide IgG is significantly stronger in cohorts vaccinated with conjugate meningococcal vaccines when compared to plain meningococcal vaccines (Findlow et al., 2009; Lieberman et al., 1996; Sikkema et al., 2000). Meningococcal polysaccharides are TI-2 antigens which characteristically produce antibodies of a lower affinity when compared to TD antigens (Mond et al., 1995; Parker, 1993). Protein TD antigens, such as tetanus toxoid (TT), are often conjugated to meningococcal polysaccharides to improve vaccine immunogenicity (Richmond et al., 1999b). In this way, more of the antibodies induced by conjugate vaccination contributes to the SBA titre compared to vaccination with the plain polysaccharides alone thus improving the correlation between the two. In one study, the R value improved from 0.45, in those vaccinated with the plain polysaccharide, to 0.77, in those vaccinated with the conjugate vaccine (Findlow et al., 2009).

Neither the standard nor modified anti-polysaccharide IgG ELISAs distinguish between the different IgG subclasses (IgG1, IgG2, IgG3, and IgG4), which vary significantly in their ability to activate complement (Brüggemann et al., 1987; Gadjeva et al., 2008; Garred et al., 1989). The inclusion of IgG subclasses that do not fix complement to the total concentration of antibody response to vaccination may also contribute to the poor correlation seen between SBA titres and the concentration of anti-polysaccharide IgG. Although the antibody subclass response to meningococcal vaccines has been assessed to some degree, the relationship between the concentration of anti-polysaccharide IgG subclasses and h/rSBA titres has not been investigated.

The bactericidal activity of vaccinee sera in the presence of human or rabbit complement correlate poorly (Findlow et al., 2009; Gill et al., 2011a). As previously discussed, there is some evidence that human antibodies differentially activate human and rabbit complement (Griffiss and Goroff, 1983; Mandrell et al., 1995; Santos et al., 2001; Zollinger and Mandrell, 1983). I hypothesise that difference in the ability of antibody subclasses to activate human and rabbit complement differentially skew SBA titres and account for the poor correlation between rSBA and hSBA titres.

3.1.1 Study Aims

The aim of this study is as follows:

i. To assess the relationship between the concentration of meningococcal polysaccharide-specific antibody subclasses and SBA titres with either human or rabbit serum.

Knowledge of the differences, if any, in which anti-polysaccharide antibody subclasses correlate with rSBA and hSBA titres may highlight important mechanisms driving the poor correlation between both assays.

3.1.1.1 Informed Consent

All testing performed in this study on clinical trial samples is covered by the consent given by the trial subjects. Furthermore, the sponsors have taken all necessary measures to ensure that the informed consent from any subject participating in this project has been obtained. Additionally, the Sponsors have taken all necessary measures to ensure that the sample information does not include any personal information (i.e., the samples have been anonymized).

3.2 Anti-MenACWY Antibody Subclass Composition – Pilot Study

To ascertain the predominant antibody subclass response to meningococcal polysaccharide vaccination, the proportion of IgG1, IgG2, IgG3, IgG4 and IgM present in affinity-purified anti-MenACWY antibody isolated from the plasma of eleven adult volunteers was assessed using a Ready-SET-Go!® Human IgM ELISA kit (eBioscience) and IgG Subclass ELISA Kit (Thermo Fisher Scientific) (***Figure 3. 1***). Plasma samples were taken at least one month post-vaccination with a quadrivalent plain polysaccharide (serogroups A, C, W-135 and Y) meningococcal vaccine (Mencevax™; ***Table 3.1***). The presence of other antibody subclasses was not assessed. Anti-MenACWY antibody was purified from anti-TT antibody-depleted plasma by affinity chromatography by injection through a 5ml MenACWY-TT-conjugated Sepharose column as described in ***Chapter 2: Section 2.2.3***.

On average, the most predominant antibody subclass present in the purified anti-MenACWY antibody was IgG2 (38.7% of total antibody) followed by IgM (23.3%), IgG1 (19.9%), IgG3 (10.2%) and IgG4 (8.2%) (***Figure 3. 1***).

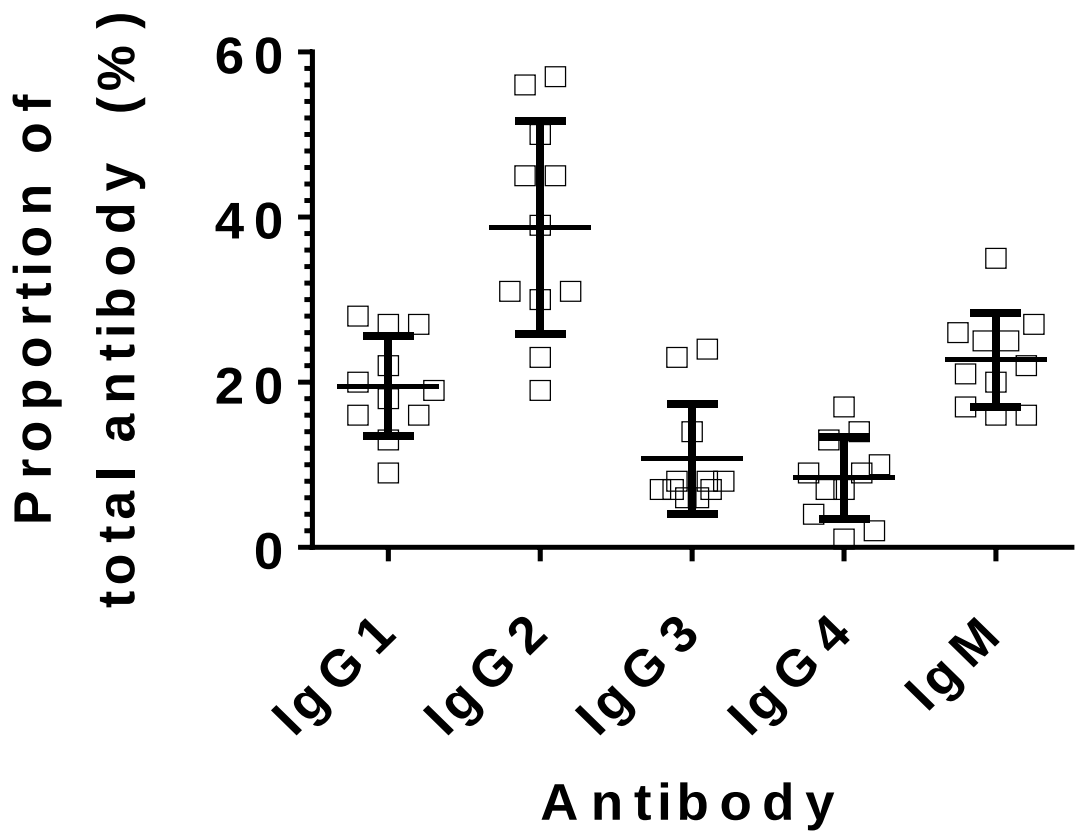


Figure 3. 1 Antibody Composition of Purified Anti-MenACWY Antibodies
Meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific antibody was affinity-purified from the plasma of eleven adult individuals. Plasma samples were taken at least one month post-vaccination with a quadrivalent plain polysaccharide (serogroups A, C, W-135 and Y) meningococcal vaccine (Mencevax™). The concentration of IgG1, IgG2, IgG3, IgG4 and IgM present in the antibody purified from each individual was assessed by running each sample (in duplicate) though a commercial IgM ELISA kit and a commercial IgG subclass ELISA kit. The proportion of each antibody subclass in each sample was calculated as the percentage of total antibody concentration (e.g. the concentration of IgG1 antibody present in the sample over the concentration of IgG1 plus IgG2 plus IgG3 plus IgG4 plus IgM present in the sample). The mean proportion of each antibody subclass present in purified meningococcal polysaccharide-specific antibody was calculated as 19.9±5.9% IgG1, 38.7±12.3% IgG2, 10.2±6.7% IgG3, 8.2±4.8% IgG4 and 23.3±5.7% IgM. Each point represents the average proportion of an antibody subclass present in the antibody purified from one individual. The errors bars represent the mean proportion (middle line) with the standard deviation (top and bottom lines) of an antibody subclass.

Table 3. 1 Cohort Details

The concentration (µg/mL) or proportion (% of total antibody) of meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific IgG1, IgG2 and IgM antibody was measured in the serum of individuals at least one month or four months post-vaccination with either a plain (Mencevax™) or a TT-conjugated (Nimenrix™) quadrivalent meningococcal polysaccharide (serogroups A, C, W-135 and Y) vaccine. Sample were taken as part of two clinical trials (identifiers: NCT00427908 and NCT00718666) as described by Vesikaria et al., (2012) and Klein et al., (2013) or as part of this project. The details of each cohort are listed below.

Clinical Trial (identifier)	***Vaccine***	***Number of Doses***	***Sample Taken***	***Number of Samples***	***Age at vaccination (months)***		***Reference***
					Mean	*Range*	
NCT00427908	Mencevax™	1	One month post vaccination	22	65	16-125	(Vesikari et al., 2012)
	Nimenrix™	1		28	41	15-131	
NCT00718666		1	Four months post final vaccination	23	12		(Klein et al., 2013)
		2		26	9 (first dose); 12 (second dose)		
N/A	Mencevax™	≥1	>one month post vaccination	11	Adult (18-55)		N/A

3.3 Anti-MenACWY IgG1, IgG2 and IgM Antibody ELISA

A series of ELISAs were developed to measure the serum concentration of *Neisseria meningitidis* serogroups A, C, W and Y polysaccharide-specific IgG1, IgG2 and IgM antibodies in individuals vaccinated with a TT-conjugated or a plain quadrivalent meningococcal polysaccharide vaccine. The details of each cohort run through each of the assays are listed in ***Table 3.1***. Firstly, a large quantity of anti-MenACWY antibody was purified and characterised for use in each assay as the standard to be used to interpolate the concentration of meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific IgG1, IgG2 and IgM antibody present in serum. To ascertain whether human antibody subclasses contribute differentially in SBAs using either human or rabbit serum as the source of complement, the concentration of meningococcal polysaccharide specific IgG1, IgG2 and IgM antibody in a sample will be correlated with the corresponding hSBA or rSBA titres achieved for that sample with each serogroup.

3.3.1 Isolation of Anti-MenACWY Antibody Standard

Anti-MenACWY antibody was isolated by affinity chromatography from 500ml pooled plasma, taken from fourteen adult individuals previously vaccinated with a quadrivalent meningococcal polysaccharide vaccine. These plasma samples were taken at least one month post-vaccination with a quadrivalent plain polysaccharide (serogroups A, C, W-135 and Y) meningococcal vaccine (Mencevax™). The plasma was first depleted of anti-TT antibody by injection through a 5ml TT-conjugated Sepharose column as described in ***Chapter 2: Section 2.2.3***. Roughly 25.5mg of anti-TT antibody and 15.3mg anti-MenACWY antibody were isolated in total. The purity and composition of the purified antibody standard was assessed by SDS-PAGE (***Figure 3.2***). SDS-PAGE analysis showed that the purified anti-MenACWY antibody standard consisted of both IgG and IgM antibody subclasses (***Figure 3. 2***).

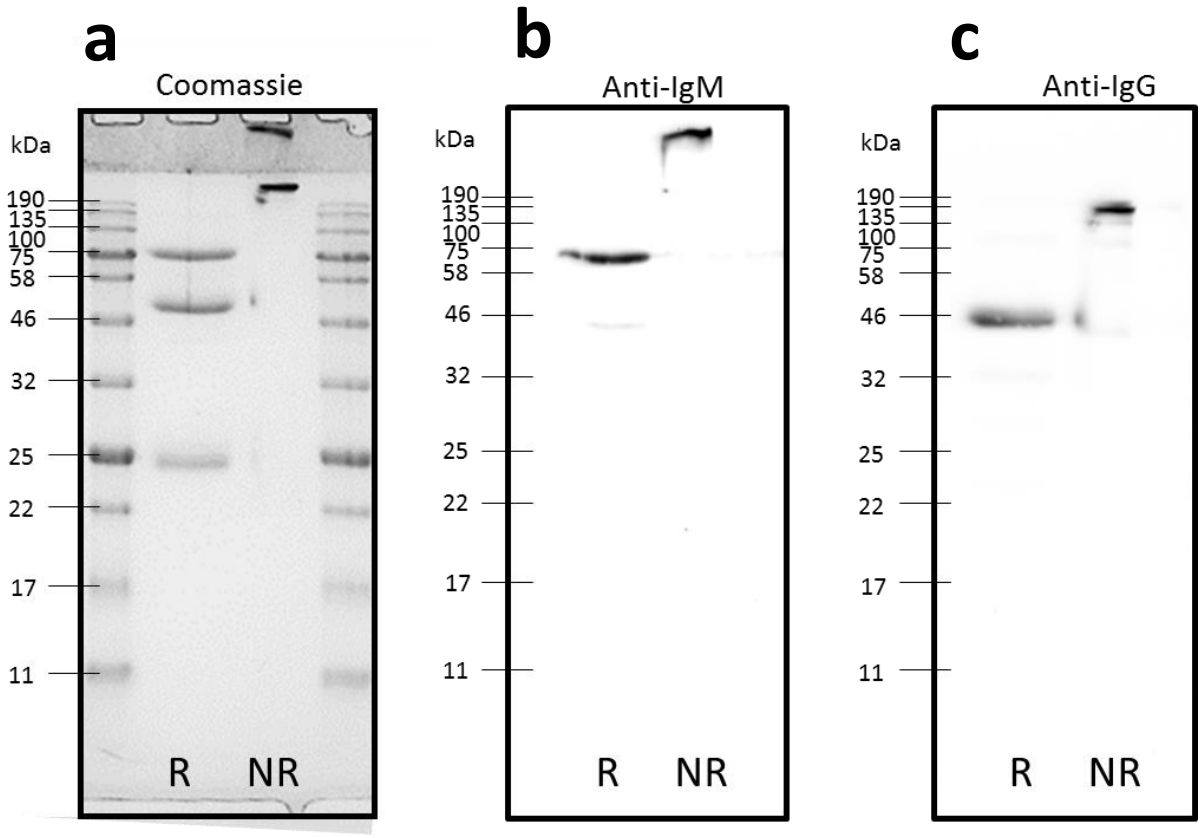


Figure 3. 2 SDS-PAGE Analysis of Anti-MenACWY Antibody Standard

A meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific antibody standard was affinity-purified from the (pooled) plasma of fourteen adult individuals. Plasma samples were taken at least one month post-vaccination with a quadrivalent plain polysaccharide (serogroups A, C, W-135 and Y) meningococcal vaccine (Mencevax™). The purity and composition of the purified antibody was assessed by SDS-PAGE. ***a***, 200ng of the purified antibody was run through a 15% polyacrylamide gel under non-reducing (NR) and reducing conditions (R) and stained with coomassie Brilliant Blue dye. Under non-reducing conditions, the purified antibody appeared as two distinct bands: one of which did not migrate through the stacking gel and one of which migrated above the 190kDa molecular weight marker. Under reducing conditions the purified antibody appeared as three bands with apparent molecular weights of 74.4kDa, 46.5kDa and 23.0kDa. These data suggested the presence of both IgG and IgM antibody subclasses in the purified antibody standard. ***b***, By western blot analysis using a donkey anti-human IgM HRP conjugate antibody, the larger band (in the non-reducing lane) and the 74.4kDa band (in the reduced lane) were positive for IgM confirming the presence of IgM in the purified antibody standard. ***c***, By western blot analysis using a donkey anti-human IgG HRP conjugate antibody, the smaller band (in the non-reducing lane) and 46.5kDa band (in the reducing lane) were both positive for IgG confirming the presence of IgG in the purified antibody standard.

The reactivity of the purified anti-MenACWY antibody standard to TT protein and MenACWY polysaccharides was assessed by ELISA (***Figure 3. 3***). The anti-MenACWY antibody bound well to MenACWY polysaccharides whereas minimal binding to TT protein could be detected confirming efficient depletion.

3.3.2 Antibody Composition of Anti-MenACWY Antibody Standard

The concentration of IgM, IgG1, IgG2, IgG3 and IgG4 present in the anti-MenACWY antibody standard was measured using a Ready-SET-Go! ® Human IgM ELISA kit (eBioscience) and IgG Subclass ELISA Kit (Thermo Fisher Scientific) (***Figure 3. 4*** and ***Figure 3. 5***). The anti-MenACWY antibody standard was run through both assays at several dilutions. The composition of the anti-MenACWY antibody standard was calculated as 335.2±26.4µg/ml IgG1, 534±31.6µg/ml IgG2, 57.5±7.5µg/ml IgG3, 77.7±5.2µg/ml IgG4 and 430.5±39.9µg/ml IgM (***Figure 3. 6***).

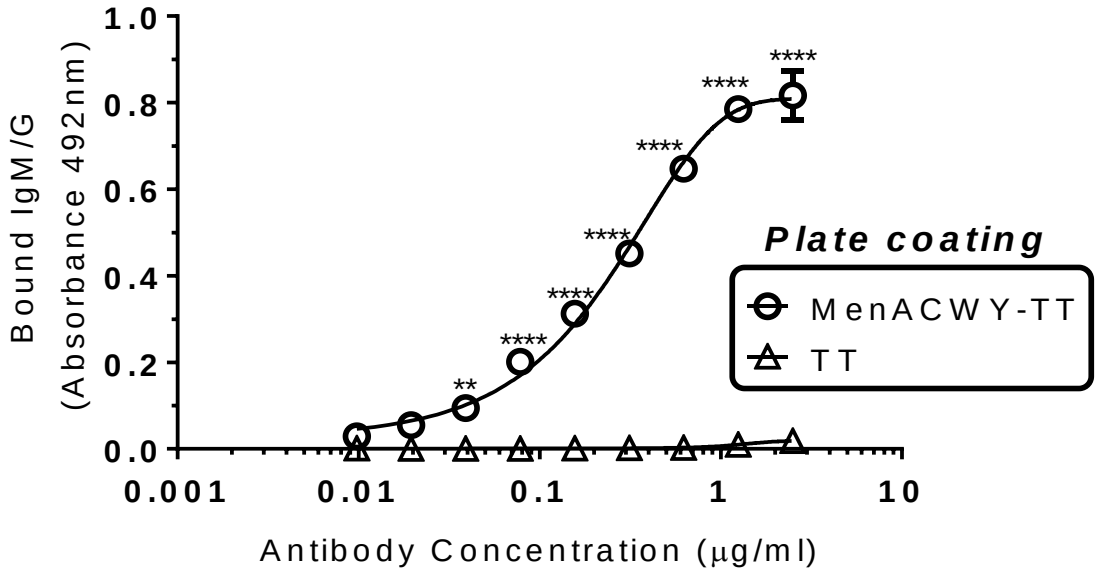


Figure 3. 3 Anti-MenACWY Antibody Standard Reactivity (ELISA)

A meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific antibody standard was affinity-purified from the (pooled) plasma of fourteen adult individuals. The antibody was purified by injection of the plasma over a MenACWY-tetanus toxoid (TT)-conjugated Sepharose column. To ensure the specificity of the purified antibody was to the meningococcal polysaccharides and not also to the TT conjugate, the plasma was first depleted of antibody specific to the TT conjugate by injection over a TT-conjugated Sepharose column prior to injection over the MenACWY-TT-conjugated Sepharose column. The reactivity of the purified antibody to TT and MenACWY-TT was assessed by ELISA. A titration (2.5-0.01μg/ml) of the purified antibody standard was incubated on ELISA plates coated with the TT conjugate or MenACWY-TT in duplicate. Subsequent IgG/M antibody binding was detected with a goat anti-human IgG/IgM HRP conjugate antibody. By two-way ANOVA multiple comparison statistical analysis comparing IgG/IgM antibody binding (absorbance 492nm) to both coatings at each concentration of the purified antibody standard, significantly more IgG/IgM binding was detected to wells coated with MenACWY-TT (open circles) compared to wells coated with TT (open triangles). These data confirmed that the specificity of the purified antibody was to the meningococcal polysaccharides and not the TT conjugate. Each point represents the average absorbance achieved for each coating at each concentration of the purified antibody standard. The errors bars represent the standard deviation. The asterisks indicate the statistical significance of the difference between the average absorbance achieved for each coating at each concentration of the purified antibody standard (*** = P value<0.001; **** = P value<0.0001).

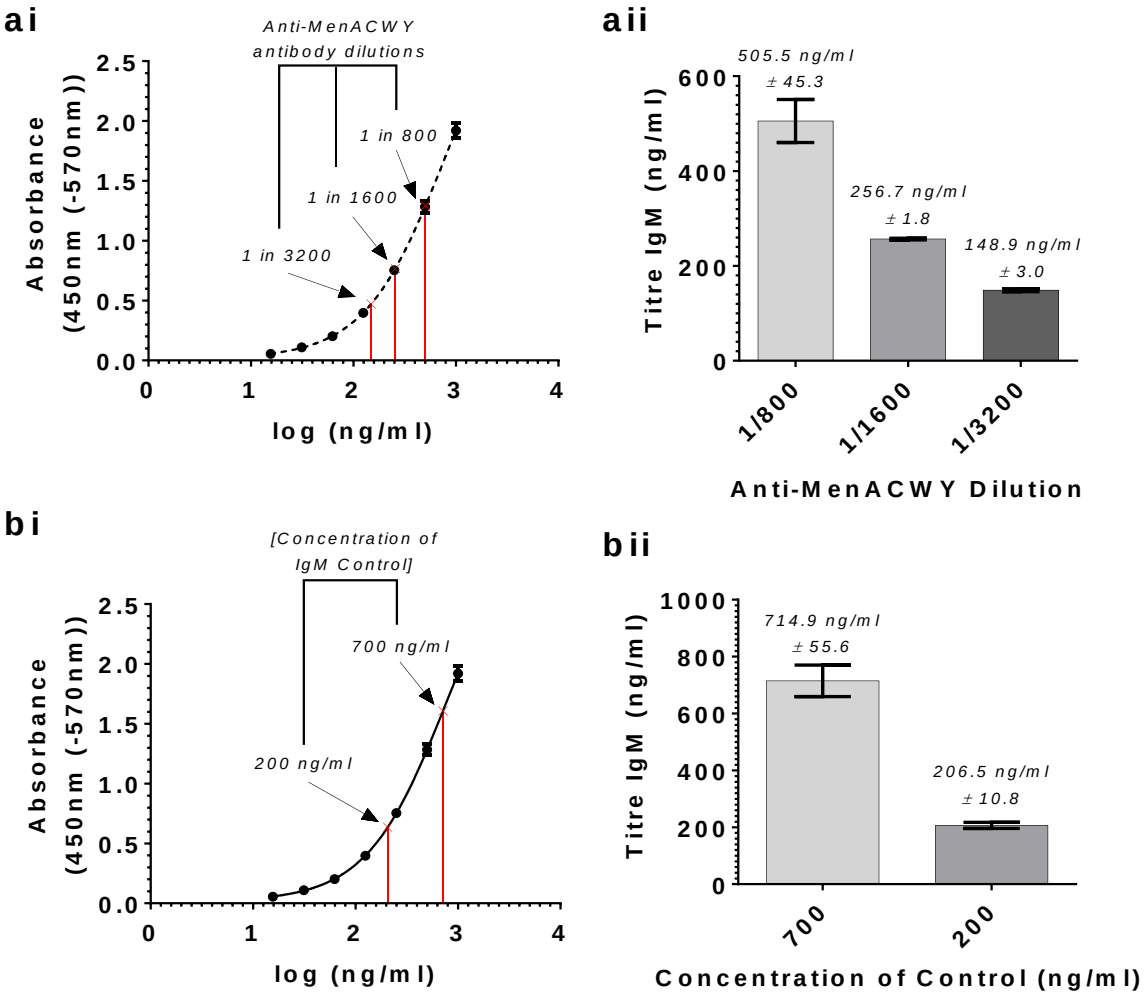


Figure 3. 4 Anti-MenACWY Antibody Standard IgM Concentration (ELISA)

A meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific antibody standard was affinity-purified from the (pooled) plasma of fourteen adult individuals. The concentration of IgM present in the purified antibody standard was assessed using a Ready-SET-Go!® Human IgM ELISA kit (eBioscience). ***ai***, The purified antibody standard was run through the assay at three different dilutions in duplicate and the concentration of IgM at each dilution was interpolated using the IgM antibody standard provided with the assay. Each point (solid circles) represents the average absorbance achieved at each concentration of the IgM antibody standard provided with the assay. The errors bars represent the standard deviation. The dotted line represents the standard curve model used to interpolate the concentration of IgM present in the three dilution of the purified antibody standard (non-linear regression; sigmoidal, 4PL standard curve). The average absorbance and interpolated concentration of each dilution of the purified antibody standard is shown as a red vertical line. ***aii***, The average interpolated concentration and standard deviation (error bars) of IgM present at each dilution of the purified antibody standard is shown. ***bi***, Two concentrations (700ng/ml and 200ng/ml) of an in-house purified human IgM were run through the same Ready-SET-Go!® Human IgM ELISA kit as a control for the assay. The absorbance achieved with each control is shown as a red vertical line. ***bii***, The average interpolated concentration and standard deviation (error bars) of each control is shown.

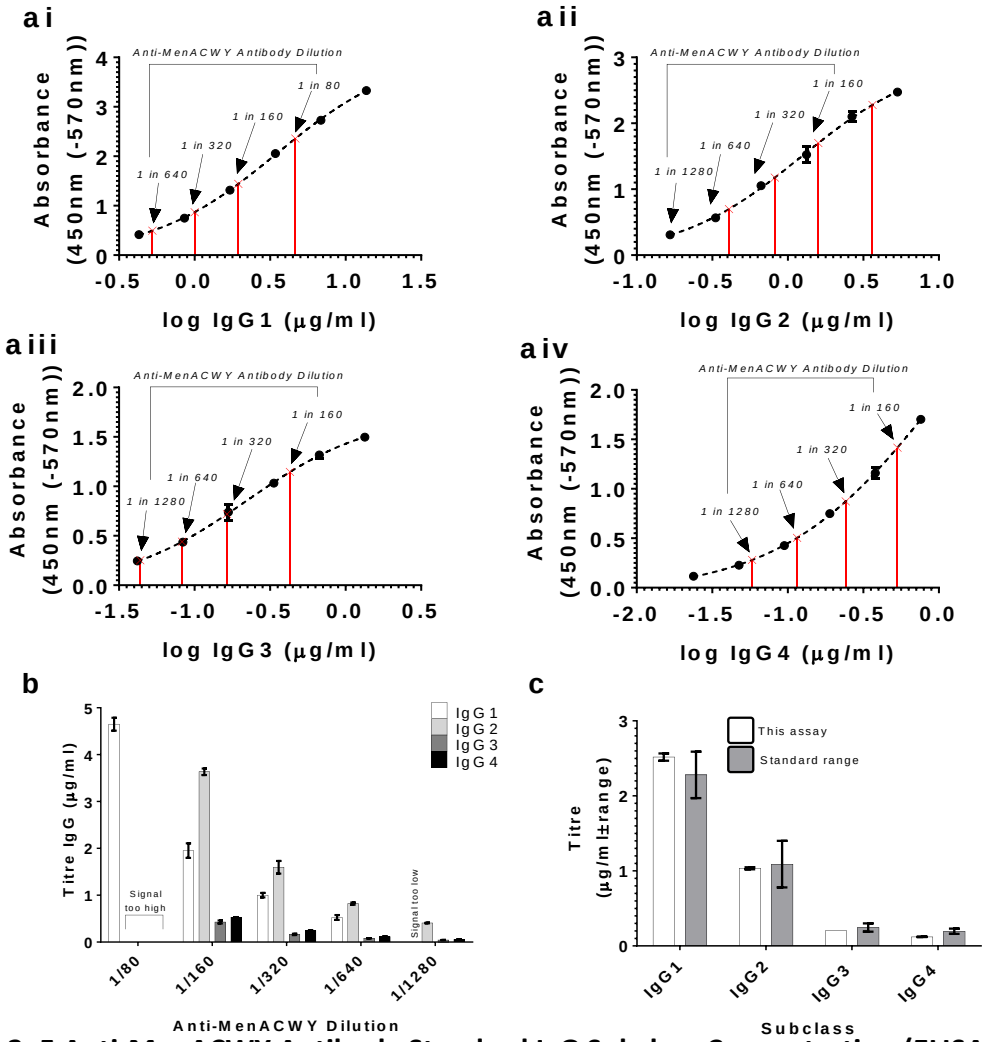


Figure 3. 5 Anti-MenACWY Antibody Standard IgG Subclass Concentration (ELISA)

A meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific antibody standard was affinity-purified from the (pooled) plasma of fourteen adult individuals. The concentration of IgG1 (***ai***), IgG2 (***aii***), IgG3 (***aiii***) and IgG4 (***aiv***) present in the purified antibody standard was assessed using a IgG Subclass ELISA Kit (Thermo Fisher Scientific). The purified antibody standard was run through the assay at five different dilutions in duplicate and the concentration of IgG1, IgG2, IgG3 and IgG4 at each dilution was interpolated using the IgG1, IgG2, IgG3 or IgG4 antibody standards provided with the assay. Each point (solid circles) represents the average absorbance achieved at each concentration of the antibody standards provided with the assay. The errors bars represent the standard deviation. The dotted line represents the standard curve model used to interpolate the concentration of IgG1, IgG2, IgG3 and IgG4 present in the five dilution of the purified antibody standard (non-linear regression; sigmoidal, 4PL standard curve). Where possible, the average absorbance and interpolated concentration of each dilution of the purified antibody standard is shown as a red vertical line. ***b***, The average interpolated concentrations and standard deviations (error bars) of IgG1, IgG2, IgG3 and IgG4 present in each dilution of the purified antibody standard are shown. The dilutions of the purified antibody standard that produced too high or too low a signal to interpolate using the antibody standards provided with the assay are also indicated. ***c***, A sample with known concentrations of IgG1, IgG2, IgG3 and IgG4 (provided with the assay) was included in the assay as the control. The average concentrations and standard deviations of IgG1, IgG2, IgG3 and IgG4 in the control sample interpolated in this assay are shown (open bars). The known concentrations and standard deviations (the details of which were provided by the supplier of the assay) of IgG1, IgG2, IgG3 and IgG4 in the control sample are also shown for comparison (filled bars).

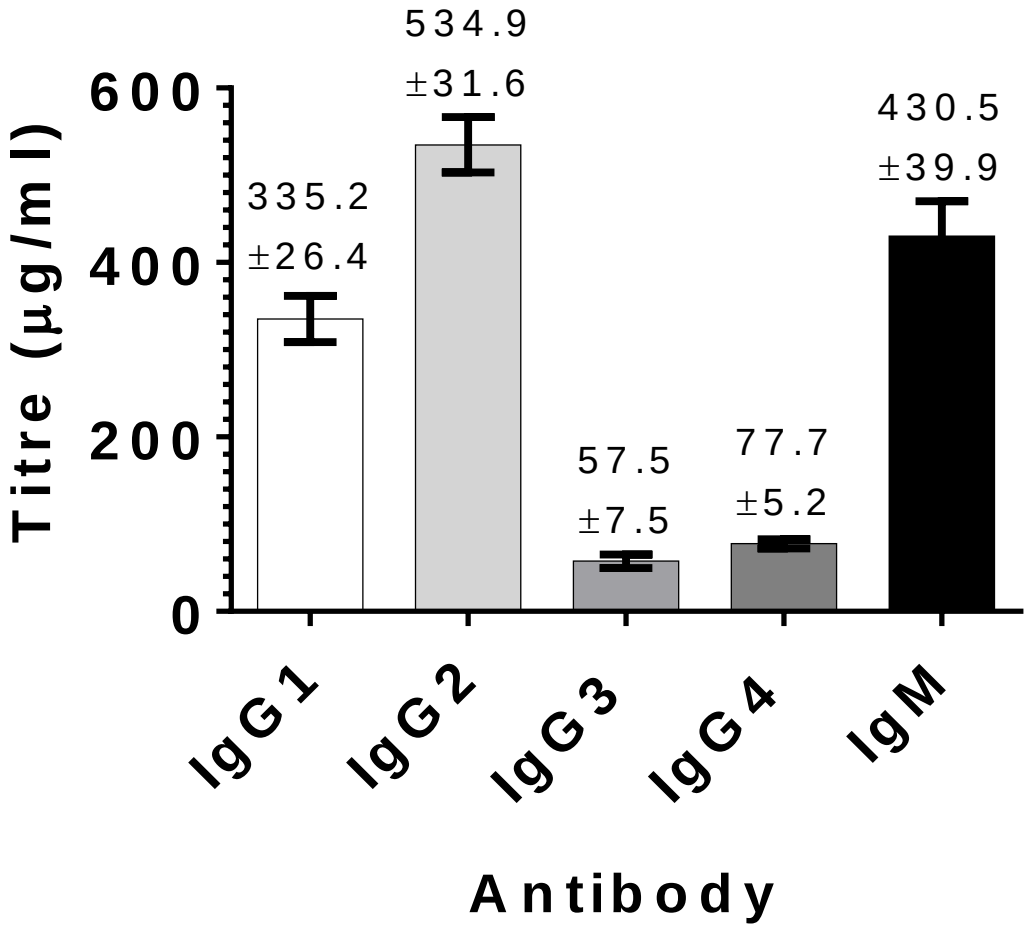


Figure 3. 6 Antibody Subclass Composition of the Anti-MenACWY Antibody Standard
A meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific antibody standard was affinity-purified from the (pooled) plasma of fourteen adult individuals. Plasma samples were taken at least one month post-vaccination with a quadrivalent plain polysaccharide (serogroups A, C, W-135 and Y) meningococcal vaccine (Mencevax™). The concentration of IgM, IgG1, IgG2, IgG3 and IgG4 present in the purified antibody standard was assessed using a Ready-SET-Go!® Human IgM ELISA kit and a IgG Subclass ELISA Kit (detailed in **Figure 3.4** and **Figure 3.5**, respectively). The purified antibody standard was calculated to consist of 335.2µg/ml IgG1, 534µg/ml IgG2, 57.5µg/ml IgG3, 77.7µg/ml IgG4 and 430.5µg/ml IgM. Each bar represents the average interpolated concentration and standard deviation (error bars) of each antibody subclass present in the purified antibody standard assessed by each assay at three (IgM) or five (IgG) different dilutions.

3.3.3 Intra-Assay and Inter-Assay Coefficients of Variation and Lower Limits of Detection

In order to ascertain the precision of each of the anti-MenACWY IgG1, IgG2 and IgM ELISAs, the intra-assay and inter-assay coefficients of variation were assessed. The intra-assay coefficient of variation describes the percentage of variation the interpolated value a sample is assigned within the same experiment/plate in an assay whereas the inter-assay coefficient of variation describes the percentage of variation the interpolated value a sample is assigned between experiments/plates in an assay.

The intra-assay coefficients of variation of anti-MenACWY IgG1, IgG2 and IgM assays were calculated by running two pooled samples through each assay seven times in duplicate (***Table 3. 2a***). The two pooled samples were created by pooling equal volumes of 10 different serum samples (20 samples in total) taken one month post-vaccination with Nimernrix™ as part of the clinical trial NCT00427908 (***Table 3.1***). Each of the seven repeats were run on the same assay plate and antibody concentration interpolated from one antibody standard. The coefficient of variation was calculated as the standard deviation divided by mean titre and expressed as the percentage of variation. The inter-assay coefficients of variation of anti-MenACWY IgG1, IgG2 and IgM assays were calculated by running two samples (pools of 10 individual samples) through each assay five times in duplicate (***Table 3. 2b***). Each of the five repeats were run on the separate assay plates and antibody concentration interpolated from separate antibody standards. The coefficient of variation was calculated as the standard deviation divided by mean titre and expressed as the percentage of variation. Both the inter-assay and intra-assay coefficients of variation were lower than 15% indicating sufficient precision for each of the anti-MenACWY IgG1, IgG2 and IgM ELISAs.

Table 3. 2 Intra-Assay and Inter-Assay Coefficient of Variation and Lower Limits of Detection

The concentration (μg/mL) of meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific IgG1, IgG2 and IgM antibody was measured by ELISA in the serum of individuals at least one month or four months post-vaccination with either a plain (Mencevax™) or a TT-conjugated (Nimenrix™) quadrivalent meningococcal polysaccharide (serogroups A, C, W-135 and Y) vaccine as described in ***Section 2.6***. ***a***, The intra-assay coefficients of variation (%) of anti-MenACWY IgG1, IgG2 and IgM assays were calculated by running two samples (pools of 10 individual serum samples) through each assay seven times time in duplicate. The average intra-assay coefficients of variation and standard deviation (SD) for both samples is shown for each assay. ***b***, The inter-assay coefficients of variation (%) of anti-MenACWY IgG1, IgG2 and IgM assays were calculated by running two samples (pools of 10 individual serum samples) through each assay five times time in duplicate. The average inter-assay coefficients of variation and SD for both samples is shown for each assay. ***c***, The lower limit of detection for each anti-MenACWY IgG1, IgG2 and IgM ELISAs was interpolated from the average absorbance achieved by a negative sample run in five sequential assays plus three standard deviations. The average lower limit of detection (μg/mL) and SD is shown for each assay.

Intra-assay Coefficients of Variation (%)								
Antibody Subclass	***Serogroup A***		***Serogroup C***		***Serogroup W***		***Serogroup Y***	
	Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
IgG1	3.79	0.37	3.41	0.25	3.45	0.63	3.46	0.65
IgG2	8.29	0.01	8.54	2.02	6.27	0.95	7.04	1.25
IgM	4.10	0.45	4.63	3.56	5.96	2.56	8.34	1.91

Inter-assay Coefficients of Variation (%)								
Antibody Subclass	***Serogroup A***		***Serogroup C***		***Serogroup W***		***Serogroup Y***	
	Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
IgG1	8.17	3.00	8.22	4.56	9.88	4.65	10.14	2.00
IgG2	9.04	5.61	8.36	7.01	7.76	0.27	11.13	4.38
IgM	6.15	2.40	8.11	2.58	8.64	1.51	7.33	0.35

Lower Limit of Detection (μg/ml)								
Antibody Subclass	***Serogroup A***		***Serogroup C***		***Serogroup W***		***Serogroup Y***	
	Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
IgG1	0.16	0.03	0.17	0.03	0.17	0.03	0.15	0.07
IgG2	0.02	0.01	0.02	0.01	0.02	0.01	0.04	0.05
IgM	0.02	0.00	0.02	0.01	0.02	0.01	0.02	0.02

In order to ascertain the minimum signal of a sample in each of the anti-MenACWY IgG1, IgG2 and IgM ELISAs where a reliable value can be interpolated, the lower limits of detection were calculated. The lower limits of detection for each anti-MenACWY IgG1, IgG2 and IgM ELISAs was interpolated from the average absorbance achieved by a negative sample run in five sequential assays plus three standard deviations. (***Table 3. 2c***).

3.4 Comparison of Antibody Response to either Plain or TT-Conjugated Quadrivalent Polysaccharide Vaccination

To ascertain whether human antibody subclasses contribute differentially in SBAs using either human or rabbit serum as the source of complement, the concentration (µg/mL) of meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific IgG1, IgG2 and IgM antibody was measured in the serum of individuals one month post-vaccination with either a plain (Mencevax™; n=22) or a TT-conjugated (Nimenrix™; n=28) quadrivalent meningococcal polysaccharide (serogroups A, C, W-135 and Y) vaccine. Serum samples were taken as part of a clinical trial (identifier: NCT00427908) comparing the efficacy of both vaccines as described by Vesikaria et al., (2012). The average age of those vaccinated with Mencevax™ was 65 months (ranging from 16 to 125 months). The average age of those vaccinated with Nimenrix™ was 41 months (ranging from 15 to 131 months) (***Table 3.1***).

A summary table showing the geometric mean serum concentrations with confidence intervals (95%) of anti-MenACWY IgG1, IgG2 and IgM antibodies one month post-vaccination with either TT- conjugated or plain MenACWY polysaccharide vaccines are shown in ***Table 3.3***. Serogroup-specific antibody subclass responses one-month post plain and TT-conjugated quadrivalent meningococcal vaccination were compared by t-test analysis (***Figure 3. 7***). A significantly higher concentration of IgG1 (MenA $P < 0.001$; MenC $P < 0.01$; MenW $P < 0.01$;

MenY P < 0.05) and IgM (MenA P < 0.001; MenC P < 0.01; MenW P < 0.001; MenY P < 0.001) antibody was measured in response to the TT-conjugated quadrivalent meningococcal vaccine compared to the plain quadrivalent meningococcal vaccine for all serogroups. A significantly higher concentration of serogroup W (P < 0.001) and Y (P < 0.05)-specific IgG2 antibody and a significantly lower concentration of serogroup A (P < 0.05)-specific IgG2 antibody was measured in response to the TT-conjugated quadrivalent meningococcal vaccine.

The antibody subclass responses between serogroups, one-month post plain and TT-conjugated quadrivalent meningococcal vaccine, were compared by t-test analysis (**Figure 3. 8**). The individuals shown in **Figure 3.8** are the same as shown in **Figure 3.7**. In general, a significantly higher concentration of IgG1, IgG2 and IgM antibody was measured in response to serogroup A compared to serogroups C, W and Y for both plain and TT-conjugated quadrivalent meningococcal vaccines (P values detailed in **Figure 3.8**).

Table 3. 3 Antibody Subclass Response to Plain and TT-Conjugated Quadrivalent Meningococcal Polysaccharide Vaccines

The concentration (µg/mL) of meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific IgG1, IgG2 and IgM antibody was measured in the serum of individuals one month post-vaccination with either a plain (Mencevax™; n=22) or a TT-conjugated (Nimenrix™; n=28) quadrivalent meningococcal polysaccharide (serogroups A, C, W-135 and Y) vaccine. Serum samples were taken as part of a clinical trial (identifier: NCT00427908) comparing the efficacy of both vaccines as described by Vesikaria et al., (2012). The average age of those vaccinated with Mencevax™ was 65 months (ranging from 16 to 125 months). The average age of those vaccinated with Nimenrix™ was 41 months (ranging from 15 to 131 months). Geometric mean concentrations (GMC; µg/mL) with 95% confidence intervals (95% CI) are shown for each serogroup-specific antibody subclass assessed one month post-vaccination with Mencevax™ (***a***; plain polysaccharide) or Nimenrix™ (***b***; TT conjugated polysaccharide).

Mencevax™ (Plain Polysaccharide)						
Serogroup	***IgG1 (µg/mL)***		***IgG2 (µg/mL)***		***IgM (µg/mL)***	
	GMC	*95% CI*	*GMC*	*95% CI*	*GMC*	*95% CI*
A	9.67	7.68-12.17	2.12	1.06-4.26	53.23	36.03-78.64
C	5.33	3.86-7.36	0.48	0.34-0.68	7.36	6.11-8.86
W	1.91	1.48-2.46	0.19	0.16-0.22	5.88	4.94-7.00
Y	2.68	1.75-4.11	0.32	0.23-0.45	7.46	6.15-9.06

Nimenrix™ (TT-Conjugated Polysaccharide)						
Serogroup	***IgG1 (µg/mL)***		***IgG2 (µg/mL)***		***IgM (µg/mL)***	
	GMC	*95% CI*	*GMC*	*95% CI*	*GMC*	*95% CI*
A	39.03	25.05-60.82	1.37	0.91-2.07	102.1	62.59-166.6
C	15.56	10.9-22.23	0.74	0.54-1.01	10.47	7.69-14.26
W	4.51	3.28-6.21	0.35	0.27-0.47	14.99	10.82-20.78
Y	6.59	4.27-10.16	0.72	0.48-1.10	18	13.11-24.74

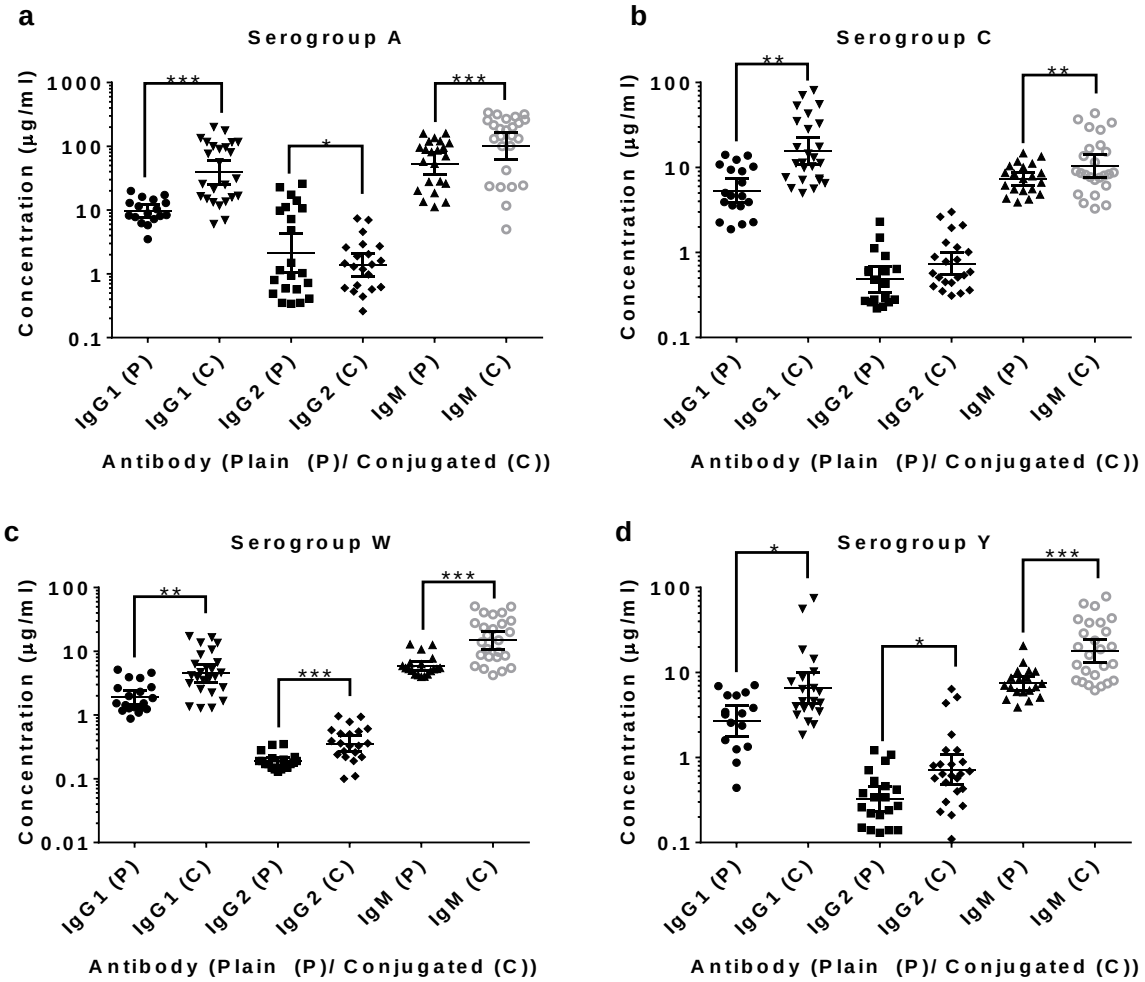


Figure 3. 7 Antibody Subclass Response to Plain versus TT-Conjugated Quadrivalent Meningococcal Polysaccharide Vaccines

The concentration (µg/mL) of meningococcal polysaccharide (serogroups A (***a***), C (***b***), W-135 (***c***) and Y (***d***)) specific IgG1, IgG2 and IgM antibody was measured in the serum of individuals one month post-vaccination with either a plain (Mencevax™; n=22) or a TT-conjugated (Nimenrix™; n=28) quadrivalent meningococcal polysaccharide (serogroups A, C, W-135 and Y) vaccine. Serum samples were taken as part of a clinical trial (identifier: NCT00427908) comparing the efficacy of both vaccines as described by Vesikaria et al., (2012). The average age of those vaccinated with Mencevax™ was 65 months (ranging from 16 to 125 months). The average age of those vaccinated with Nimenrix™ was 41 months (ranging from 15 to 131 months). Significant differences in antibody subclass concentrations between plain and TT-conjugated vaccines for each serogroup were calculated by t-test statistical analysis. The asterisks indicate the statistical significance of the difference between the serum antibody subclass concentration post vaccination with either Mencevax™ or Nimenrix™ for each of the serogroups (* = P<0.05; ** = P value <0.01; *** = P value<0.001). Each serum sample was run through each assay in duplicate. Each point represents the average concentration (µg/mL) of the serogroup-specific antibody subclass present in the serum of one individual. The errors bars represent the geometric mean concentration (µg/mL; middle line) of the serogroup-specific antibody subclass with the 95% confidence intervals (top and bottom lines). The geometric mean concentrations (µg/mL) and 95% confidence intervals for each serogroup-specific antibody subclass in each group are summarised in ***Table 3.3***.

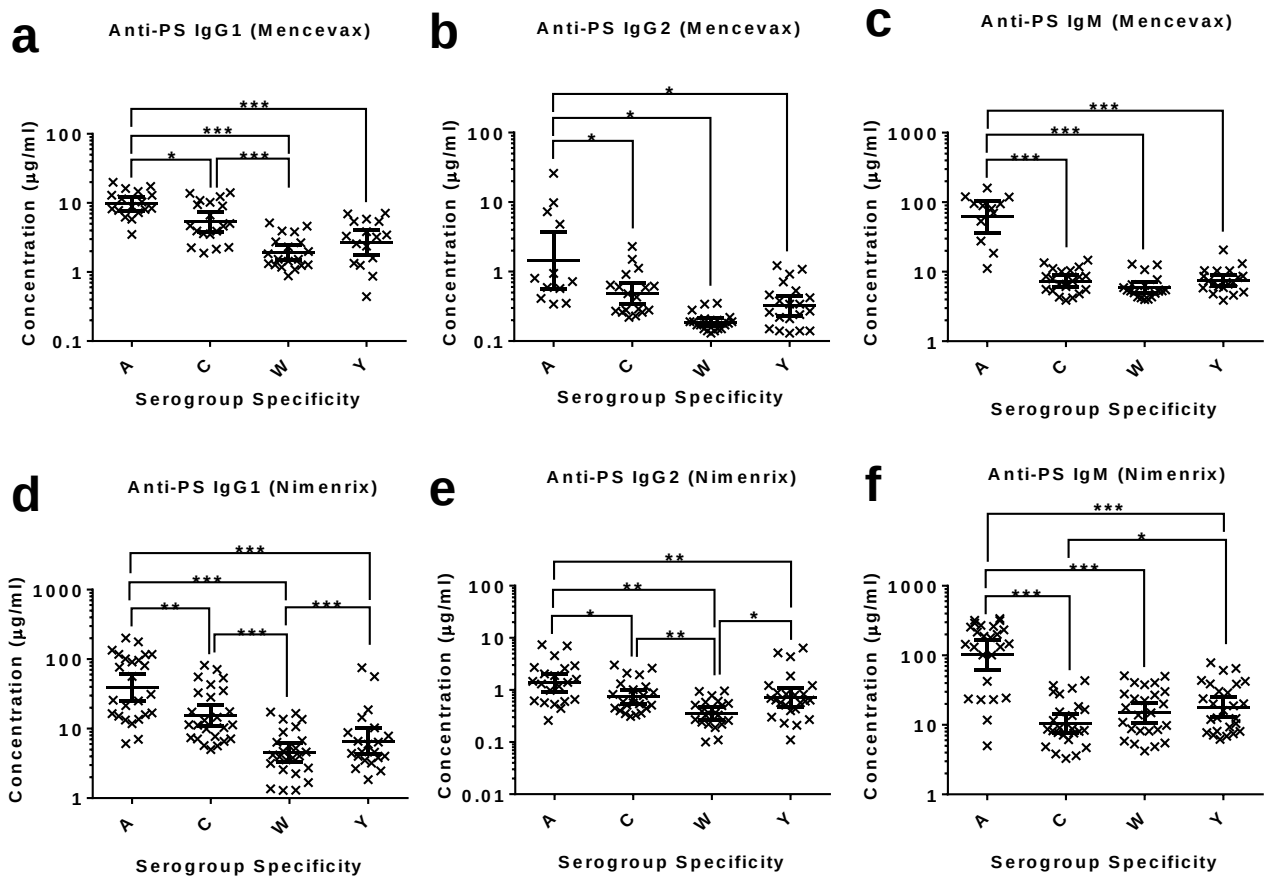


Figure 3. 8 Antibody Subclass Response to Plain and TT-Conjugated Quadrivalent Meningococcal Polysaccharide Vaccine by Serogroup

The concentration (µg/mL) of meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific IgG1, IgG2 and IgM antibody was measured in the serum of individuals one month post-vaccination with either a plain (Mencevax™; n=22) or a TT-conjugated (Nimenrix™; n=28) quadrivalent meningococcal polysaccharide (serogroups A, C, W-135 and Y) vaccine (NB. The individuals shown here are the same as shown in ***Figure 3.7***). Serum samples were taken as part of a clinical trial (identifier: NCT00427908) comparing the efficacy of both vaccines as described by Vesikaria et al., (2012). Significant differences in the antibody subclass concentrations specific for each of the four serogroups after vaccination with either Mencevax™ (***a***, ***b*** and ***c***) or Nimenrix™ (***d***, ***e*** and ***f***) were compared by t-test statistical analysis. The asterisks indicate the statistical significance of the difference between the antibody subclass concentration specific for each of the four serogroups (* = P<0.05; ** = P value <0.01; *** = P value<0.001). Each serum sample was run through each assay in duplicate. Each point represents the average concentration (µg/mL) of the serogroup-specific antibody subclass present in the serum of one individual. The errors bars represent the geometric mean concentration (µg/mL; middle line) of the serogroup-specific antibody subclass with the 95% confidence intervals (top and bottom lines). The geometric mean concentrations (µg/mL) and 95% confidence intervals for each serogroup-specific antibody subclass in each group are summarised in ***Table 3.3***.

3.4.1 Relationship between Antibody Subclasses and SBA Titres

To ascertain whether human antibody subclasses contribute differentially in SBAs using either human or rabbit serum as the source of complement, the relationship between the concentration of serogroup-specific antibody subclasses and h/rSBA titres was investigated by analysing the serum from individuals one month post vaccination with either plain or TT-conjugated quadrivalent meningococcal polysaccharide vaccines (***Figure 3. 9*** and ***Figure 3. 10***). The individuals shown in **Figure 3.9** and **Figure 3.10** are the same as shown in **Figure 3.7** and **Figure 3.8.** SBA titres of serum samples towards *Neisseria meningitidis* serogroups A (strain 3125), C (strain C11), W-135 (strain MP01240070) and Y (strain 1975) were assessed during the clinical trial NCT00427908 (ClinicalTrials.gov, 2012). SBAs were performed at GlaxoSmithKline Vaccines, Wavre, Belgium by Vesikari et al., (2012). The source of human complement used in the hSBA is a non-depleted human serum that was specifically screened to determine absence of antibodies to *Neisseria meningitidis* and absence of intrinsic toxicity. Scatter plots comparing the concentration of each serogroup-specific antibody subclass with hSBA and rSBA titres are shown in ***Figure 3. 9*** and ***Figure 3. 10***, respectively. The numbers of individuals vary due to the availability of hSBA and rSBA titres for each individual.

hSBA titres correlated most significantly with the concentration of serogroup A (strain 3125), W-135 (strain MP01240070) and Y (strain 1975)-specific IgG1 antibody in response to the TT-conjugated quadrivalent meningococcal polysaccharide vaccine only. Aside from the concentration of serogroup A (strain 3125)-specific IgG1 antibody, hSBA titres correlated poorly with the concentration of serogroup-specific IgG1, IgG2 and IgM antibody in response to the plain quadrivalent meningococcal polysaccharide vaccine. In contrast, rSBA titres correlated most significantly with the concentration of serogroups A (strain 3125), C (strain C11), W-135 (strain MP01240070) and Y (strain 1975)-specific IgM antibody in response to both the plain and TT-conjugated quadrivalent meningococcal polysaccharide vaccines.

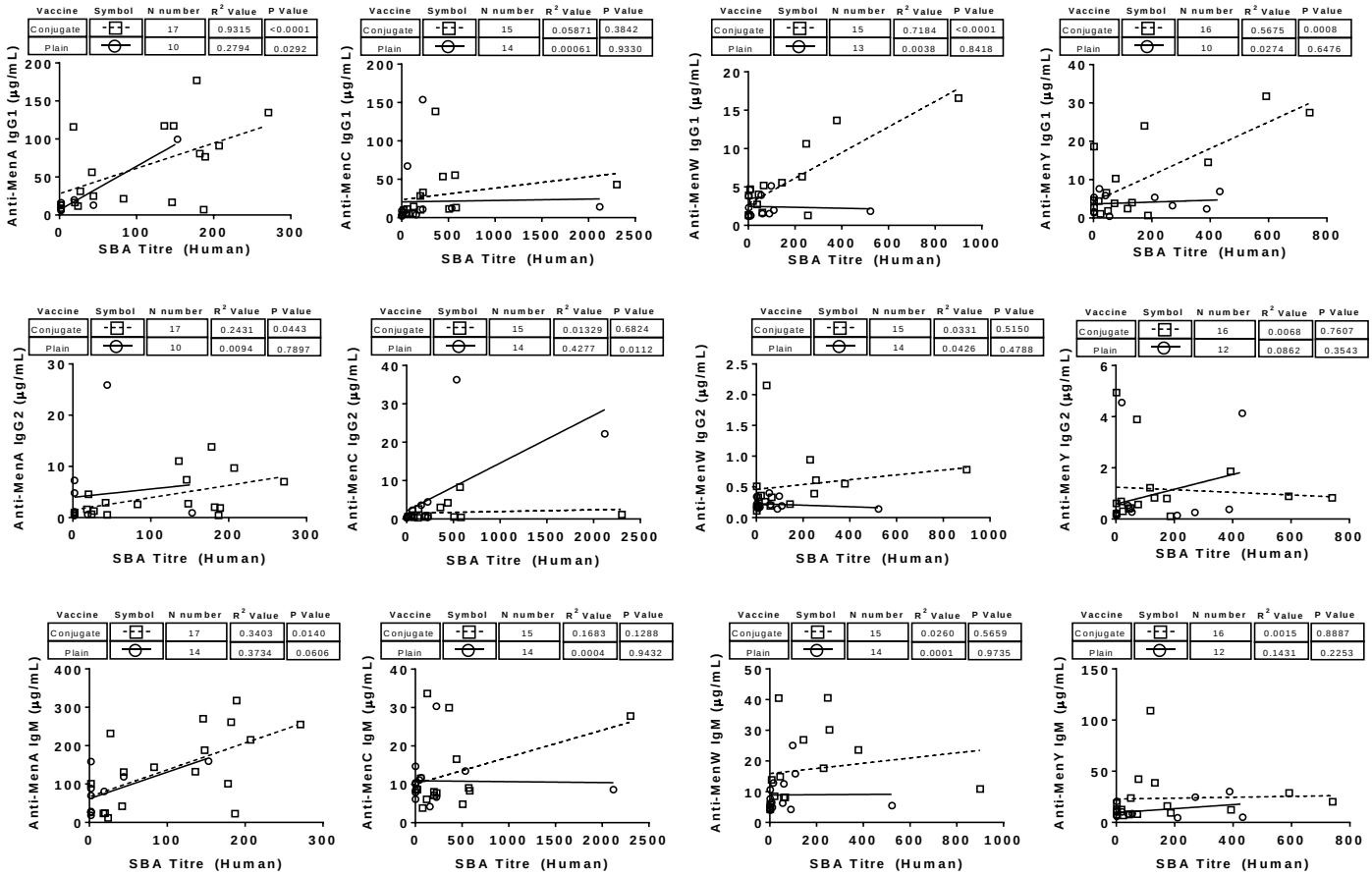


Figure 3. 9 Relationship between Antibody Subclasses and hSBA Titres

The concentration (µg/mL) of meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific IgG1, IgG2 and IgM antibody was measured in the serum of individuals one month post-vaccination with either a plain (Mencevax™) or a TT-conjugated (Nimenrix™) quadrivalent meningococcal polysaccharide (serogroups A, C, W-135 and Y) vaccine (NB. The individuals shown here are the same as shown in ***Figure 3.7*** and ***Figure 3.8***). Serum samples were taken and SBA titres (using human serum as the source of complement; hSBA titre) were assessed to each serogroup as part of a clinical trial (identifier: NCT00427908) comparing the efficacy of both vaccines as described by Vesikaria et al., (2012). Each scatter plot shows the relationship between the concentration of a serogroup-specific antibody subclass and hSBA titres one month post vaccination with either plain (open circle; solid line) or TT-conjugated (open square; dotted line) quadrivalent polysaccharide vaccines. Each point represents the average serum concentration (µg/mL) of a serogroup-specific antibody subclass and the hSBA titre to the same serogroup of one individual. The number of samples in each group are indicated in each of the scatter plots. N numbers vary due to the availability of hSBA titres for each individual. The correlation (R value) is indicated for each group in each of the scatter plots which was calculated by linear regression. The statistical significance (P value) of each correlation is also shown.

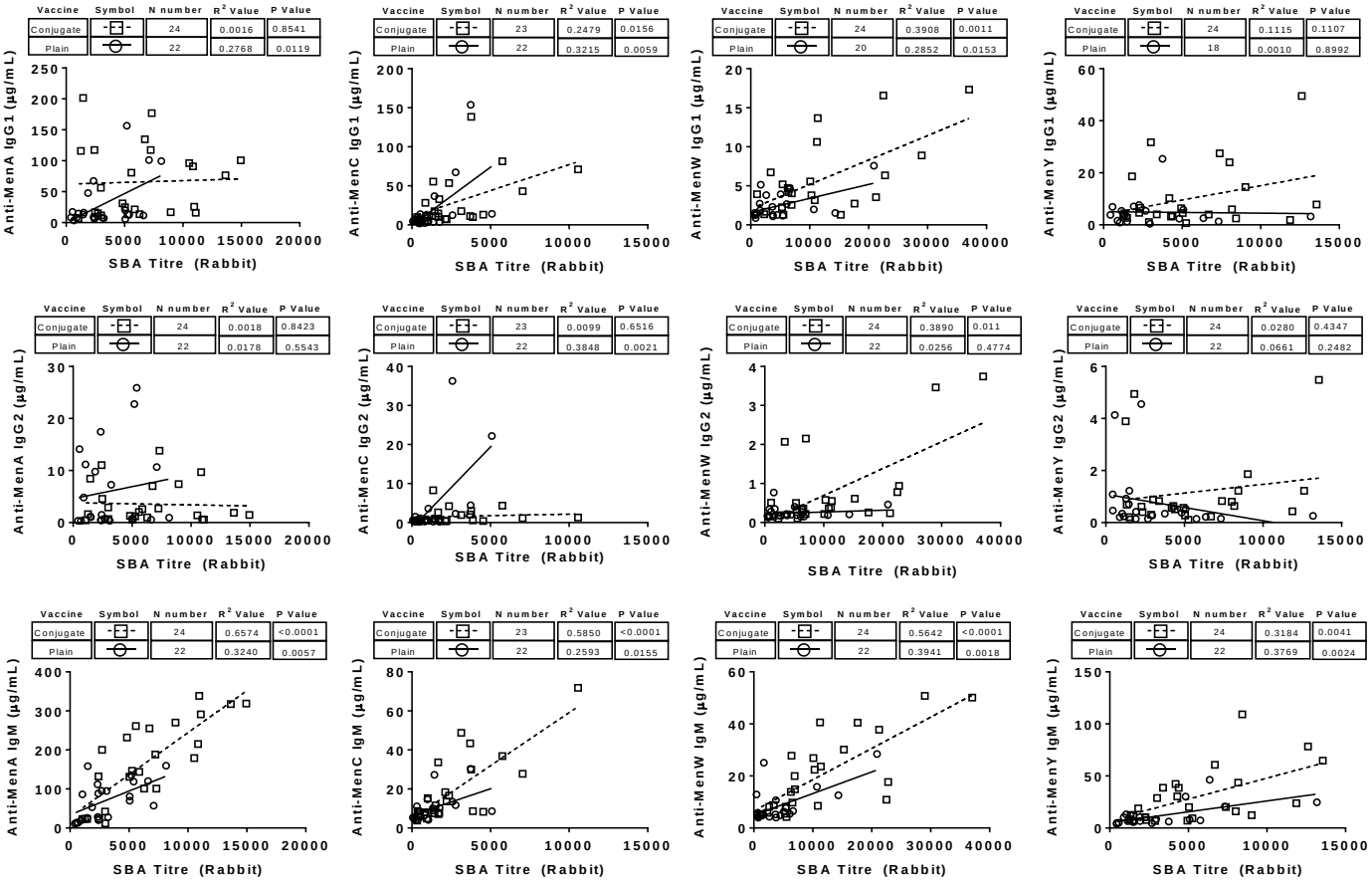


Figure 3. 10 Relationship between Antibody Subclasses and rSBA Titres

The concentration (µg/mL) of meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific IgG1, IgG2 and IgM antibody was measured in the serum of individuals one month post-vaccination with either a plain (Mencevax™) or a TT-conjugated (Nimenrix™) quadrivalent meningococcal polysaccharide (serogroups A, C, W-135 and Y) vaccine (NB. The individuals shown here are the same as shown in ***Figure 3.7, Figure 3.8*** and ***Figure 3.9***). Serum samples were taken and SBA titres (using rabbit serum as the source of complement; rSBA titre) were assessed to each serogroup as part of a clinical trial (identifier: NCT00427908) comparing the efficacy of both vaccines as described by Vesikaria et al., (2012). Each scatter plot shows the relationship between the concentration of a serogroup-specific antibody subclass and rSBA titres one month post vaccination with either plain (open circle; solid line) or TT-conjugated (open square; dotted line) quadrivalent polysaccharide vaccines. Each point represents the average serum concentration (µg/mL) of a serogroup-specific antibody subclass and the rSBA titre to the same serogroup of one individual. The number of samples in each group are indicated in each of the scatter plots. N numbers vary due to the availability of hSBA titres for each individual. The correlation (R value) is indicated for each group in each of the scatter plots which was calculated by linear regression. The statistical significance (P value) of each correlation is also shown.

3.5 Comparison of Antibody Response to either One or Two Vaccinations with TT-Conjugated Quadrivalent Polysaccharide Vaccine

To ascertain whether human antibody subclasses contribute differentially in SBAs using either human or rabbit serum as the source of complement, the concentration (µg/mL) of meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific IgG1, IgG2 and IgM antibody was measured in the serum of individuals four months post-vaccination with either one dose (n=23) or two doses of TT-conjugated (n=26) quadrivalent meningococcal polysaccharide (serogroups A, C, W-135 and Y) vaccine. Serum samples were taken as part of a clinical trial (identifier: NCT00718666) comparing the efficacy of one versus two doses of Nimenrix™ as described by Klein et al., (2013). The age of those vaccinated with one dose Nimenrix™ was 12 months. The age of those vaccinated with two doses Nimenrix™ was 9 months (first dose) and 12 months (second dose).

A summary table of geometric mean concentrations with confidence intervals (95%) of anti-MenACWY IgG1, IgG2 and IgM antibodies following either one or two doses Nimenrix™ vaccine is shown in ***Table 3.4***. Serogroup-specific antibody subclass responses four months post one versus two doses TT-conjugated quadrivalent meningococcal vaccine were compared by t-test analysis (***Figure 3. 11***). A significantly lower concentration of serogroup A-specific IgG1 ($P<0.05$), IgG2 ($P<0.05$) and IgM ($P<0.05$) and serogroup C-specific IgM ($P<0.05$) antibody was measured in response to two doses of the TT-conjugated quadrivalent meningococcal vaccine compared to one dose. In contrast, a significantly higher concentration of serogroup Y-specific IgG1 ($P<0.05$) and IgG2 ($P<0.05$) and serogroup W-specific IgG2 ($P<0.05$) antibody was measured in response to two doses of the TT-conjugated quadrivalent meningococcal vaccine compared to one dose.

Table 3. 4 Antibody Subclass Response to One and Two Doses TT-Conjugated Quadrivalent Meningococcal Polysaccharide Vaccine

The concentration (µg/mL) of meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific IgG1, IgG2 and IgM antibody was measured in the serum of individuals four months post-vaccination with either one dose (n=23) or two doses of TT-conjugated (n=26) quadrivalent meningococcal polysaccharide (serogroups A, C, W-135 and Y) vaccine. Serum samples were taken as part of a clinical trial (identifier: NCT00718666) comparing the efficacy of one versus two doses of Nimenrix™ as described by Klein et al., (2013). The age of those vaccinated with one dose Nimenrix™ was 12 months. The age of those vaccinated with two doses Nimenrix™ was 9 months (first dose) and 12 months (second dose). Geometric mean concentrations (GMC; µg/mL) with 95% confidence intervals (95% CI) are shown for each serogroup-specific antibody subclass assessed four months post-vaccination with a single dose of Nimenrix™ (***a***) or two doses of Nimenrix™ (***b***).

One Dose Nimenrix™						
Serogroup	***IgG1 (µg/mL)***		***IgG2 (µg/mL)***		***IgM (µg/mL)***	
	GMC	*95% CI*	*GMC*	*95% CI*	*GMC*	*95% CI*
A	19.29	12.94-28.74	1.09	0.55-2.18	20.5	14.04-29.94
C	9.89	7.931-12.32	0.42	0.33-0.53	7.4	5.89-9.31
W-135	4.391	3.21-6.01	0.312	0.21-0.47	7.11	5.73-8.83
Y	5.43	3.21-9.20	0.35	0.22-0.53	7.58	6.26-9.18

Two Doses Nimenrix™						
Serogroup	***IgG1 (µg/mL)***		***IgG2 (µg/mL)***		***IgM (µg/mL)***	
	GMC	*95% CI*	*GMC*	*95% CI*	*GMC*	*95% CI*
A	10.02	7.83-12.83	0.37	0.26-0.53	9.44	6.56-13.60
C	7.68	5.82-10.14	0.39	0.29-0.53	4.25	3.49-5.17
W-135	4.84	2.85-8.20	0.74	0.35-1.59	6.2	5.13-7.49
Y	11.87	6.47-21.76	1.17	0.58-2.37	6.16	5.10-7.45

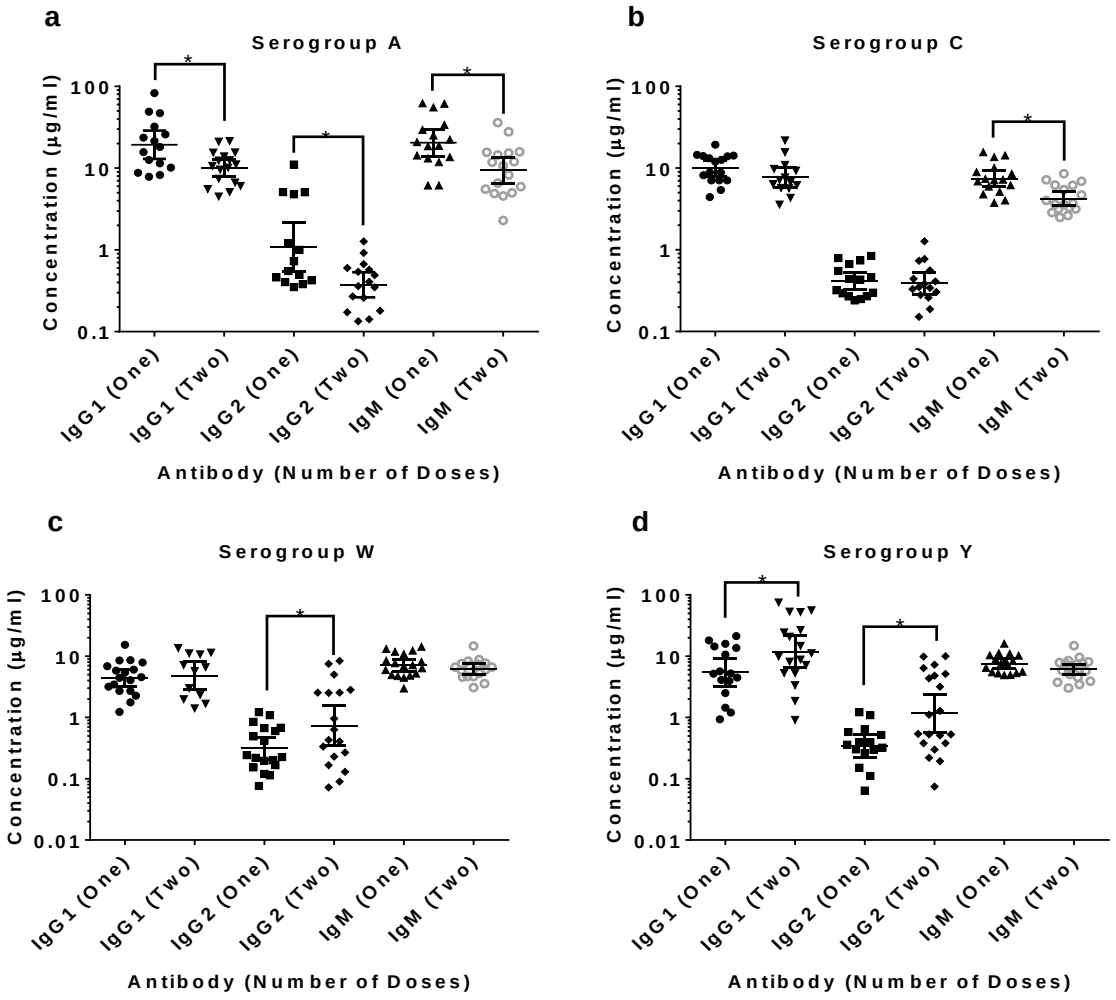


Figure 3. 11 Antibody Subclass Response to One Versus Two Doses TT-Conjugated Quadrivalent Meningococcal Polysaccharide Vaccine

The concentration (µg/mL) of meningococcal polysaccharide (serogroups A (***a***), C (***b***), W-135 (***c***) and Y (***d***)) specific IgG1, IgG2 and IgM antibody was measured in the serum of individuals four months post-vaccination with either one dose (n=23) or two doses of TT-conjugated (n=26) quadrivalent meningococcal polysaccharide (serogroups A, C, W-135 and Y) vaccine. Serum samples were taken as part of a clinical trial (identifier: NCT00718666) comparing the efficacy of one versus two doses of Nimenrix™ as described by Klein et al., (2013). The age of those vaccinated with one dose Nimenrix™ was 12 months. The age of those vaccinated with two doses Nimenrix™ was 9 months (first dose) and 12 months (second dose). Significant differences in antibody subclass concentrations between one and two doses of the Nimenrix™ vaccine for each serogroup were calculated by t-test statistical analysis. The asterisks indicate the statistical significance of the difference between the serum antibody subclass concentration post vaccination with either one dose or two doses Nimenrix™ for each of the serogroups (* = P<0.05; ** = P value <0.01; *** = P value<0.001). Each serum sample was run through each assay in duplicate. Each point represents the average concentration (µg/mL) of the serogroup-specific antibody subclass present in the serum of one individual. The errors bars represent the geometric mean concentration (µg/mL; middle line) of the serogroup-specific antibody subclass with the 95% confidence intervals (top and bottom lines). The geometric mean concentrations (µg/mL) and 95% confidence intervals for each serogroup-specific antibody subclass in each group are summarised in ***Table 3.5***.

The antibody subclass responses between serogroups, four months post one and two doses TT-conjugated quadrivalent meningococcal vaccine, were compared by t-test analysis (***Figure 3. 12***). The individuals shown in **Figure 3.12** are the same as shown in **Figure 3.11**. Following one dose of the vaccine, a significantly higher concentration of IgG1, IgG2 and IgM antibody was measured in response to serogroup A compared to serogroups C, W and Y.

3.5.1 Relationship between Antibody Subclasses and SBA Titres

To ascertain whether human antibody subclasses contribute differentially in SBAs using either human or rabbit serum as the source of complement, the relationship between the concentration of serogroup-specific antibody subclasses and h/rSBA titres was investigated following one and two doses TT-conjugated MenACWY polysaccharide vaccine (***Figure 3. 13*** and ***Figure 3. 14***). The individuals shown in **Figure 3.11** and **Figure 3.12** are the same as shown in **Figure 3.13** and **Figure 3.14**. There was no significant difference between the correlations of serogroup-specific antibody subclasses and h/rSBA titres of individuals vaccinated with either one or two doses. As such, the data from these two cohorts were combined to improve the power of this study. SBA titres of serum samples towards *Neisseria meningitidis* serogroups A (strain 3125), C (strain C11), W-135 (strain MP01240070) and Y (strain 1975) were assessed during the clinical trial NCT00718666 (ClinicalTrials.gov, 2013). SBAs were performed at either GlaxoSmithKline Vaccines, Rixensart, Belgium by Klein et al., (2013). The source of human complement used in the hSBA is a non-depleted human serum that was specifically screened to determine absence of antibodies to *Neisseria meningitidis* and absence of intrinsic toxicity. Scatter plots comparing the concentration of each serogroup-specific antibody subclass with hSBA and rSBA are shown in ***Figure 3. 13*** and ***Figure 3. 14***, respectively. The numbers of individuals vary due to the availability of hSBA and rSBA titres for each individual. Aside from the concentration of serogroup A-specific IgG1 and IgG2 antibody, hSBA titres correlated most

significantly with the concentration of serogroup-specific IgG1 and IgG2 antibody in response to one and two doses of the TT-conjugated quadrivalent meningococcal polysaccharide vaccine. Furthermore, hSBA titres correlated poorly with the concentration of serogroup-specific IgM antibody. In contrast, rSBA titres correlated most significantly with the concentration of serogroup A (strain 3125), W-135 (strain MP01240070) and Y (strain 1975) IgM antibody but correlated poorly with the concentration of serogroup-specific IgG1 and IgG2 antibody.

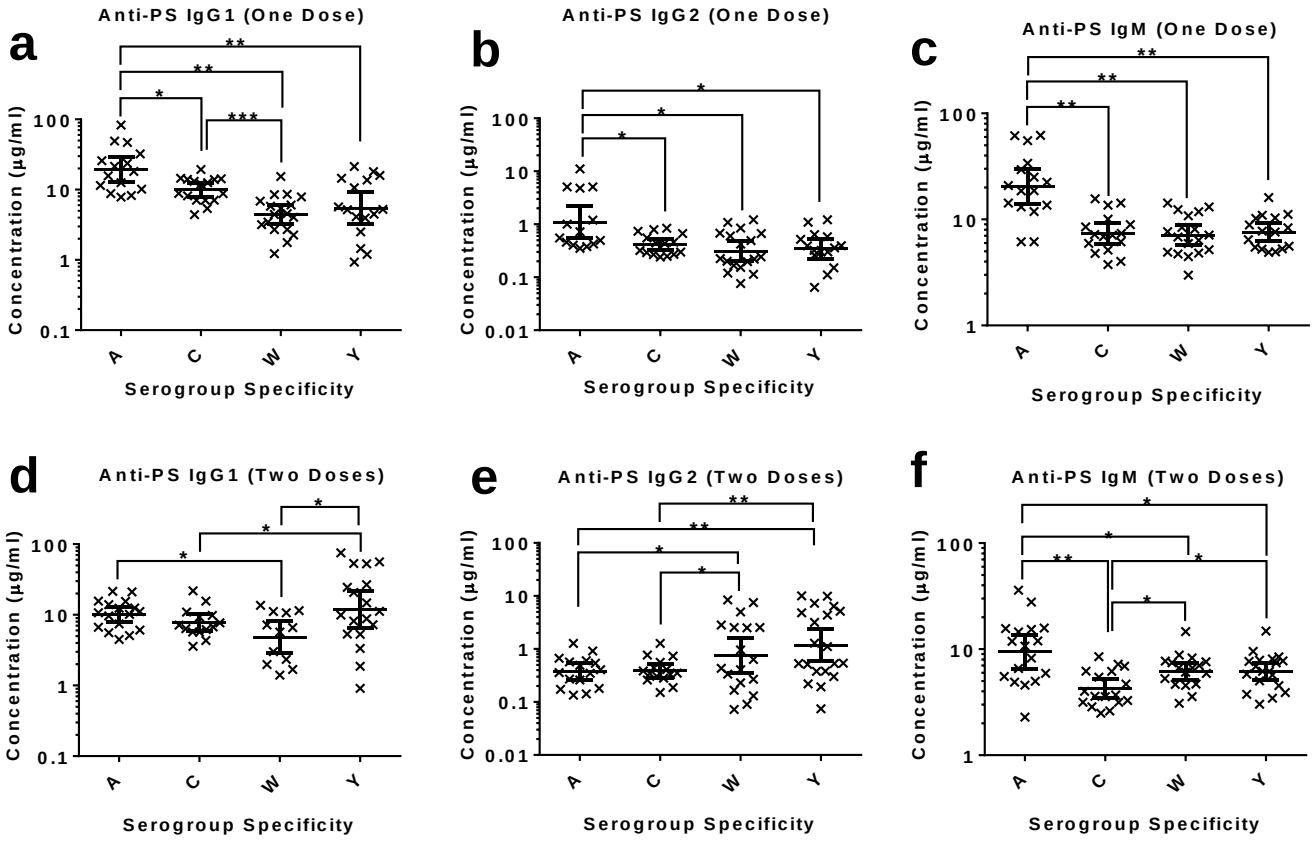


Figure 3. 12 Antibody Subclass Response to One and Two Doses TT-Conjugated Quadrivalent Meningococcal Polysaccharide Vaccine by Serogroup

The concentration (µg/mL) of meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific IgG1, IgG2 and IgM antibody was measured in the serum of individuals four months post-vaccination with either one dose (n=23) or two doses of a TT-conjugated (n=26) quadrivalent meningococcal polysaccharide (serogroups A, C, W-135 and Y) vaccine (NB. The individuals shown here are the same as shown in ***Figure 3.11***). Serum samples were taken as part of a clinical trial (identifier: NCT00718666) comparing the efficacy of one versus two doses of Nimenrix™ as described by Klein et al., (2013). Significant differences in the antibody subclass concentrations specific for each of the four serogroups after vaccination with either one dose (***a***, ***b*** and ***c***) or two doses of Nimenrix™ (***d***, ***e*** and ***f***) were compared by t-test statistical analysis. The asterisks indicate the statistical significance of the difference between the antibody subclass concentration specific for each of the four serogroups (* = P<0.05; ** = P value <0.01; *** = P value<0.001). Each serum sample was run through each assay in duplicate. Each point represents the average concentration (µg/mL) of the serogroup-specific antibody subclass present in the serum of one individual. The errors bars represent the geometric mean concentration (µg/mL; middle line) of the serogroup-specific antibody subclass with the 95% confidence intervals (top and bottom lines). The geometric mean concentrations (µg/mL) and 95% confidence intervals for each serogroup-specific antibody subclass in each group are summarised in ***Table 3.5***.

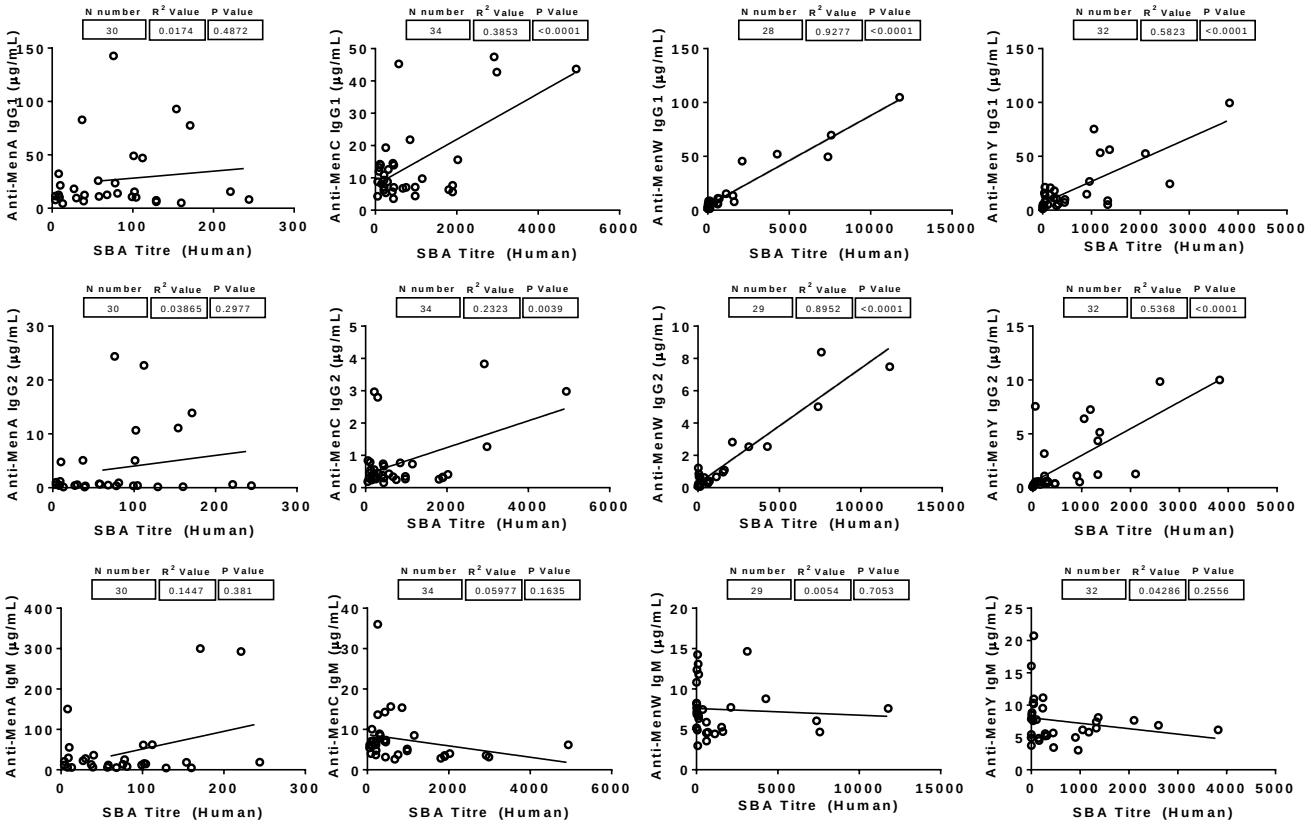


Figure 3. 13 Relationship between Antibody Subclass Response Post Vaccination with TT-Conjugated Quadrivalent Polysaccharide Vaccine and hSBA Titres

The concentration (µg/mL) of meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific IgG1, IgG2 and IgM antibody was measured in the serum of individuals four months post-vaccination with either one dose (n=23) or two doses of a TT-conjugated (n=26) quadrivalent meningococcal polysaccharide (serogroups A, C, W-135 and Y) vaccine (NB. The individuals shown here are the same as shown in ***Figure 3.11*** and ***Figure 3.12***). Serum samples were taken and SBA titres (using human serum as the source of complement; hSBA titre) were assessed to each serogroup as part of a clinical trial (identifier: NCT00718666) comparing the efficacy of one versus two doses of Nimenrix™ as described by Klein et al., (2013). Each scatter plot shows the relationship between the concentration of a serogroup-specific antibody subclass and hSBA titres four months post vaccination with one and two doses of the TT-conjugated quadrivalent polysaccharide vaccine (open circle; solid line). Each point represents the average serum concentration (µg/mL) of a serogroup-specific antibody subclass and the hSBA titre to the same serogroup of one individual. The number of samples in each group are indicated in each of the scatter plots. N numbers vary due to the availability of hSBA titres for each individual. The correlation (R value) is indicated for each group in each of the scatter plots which was calculated by linear regression. The statistical significance (P value) of each correlation is also shown.

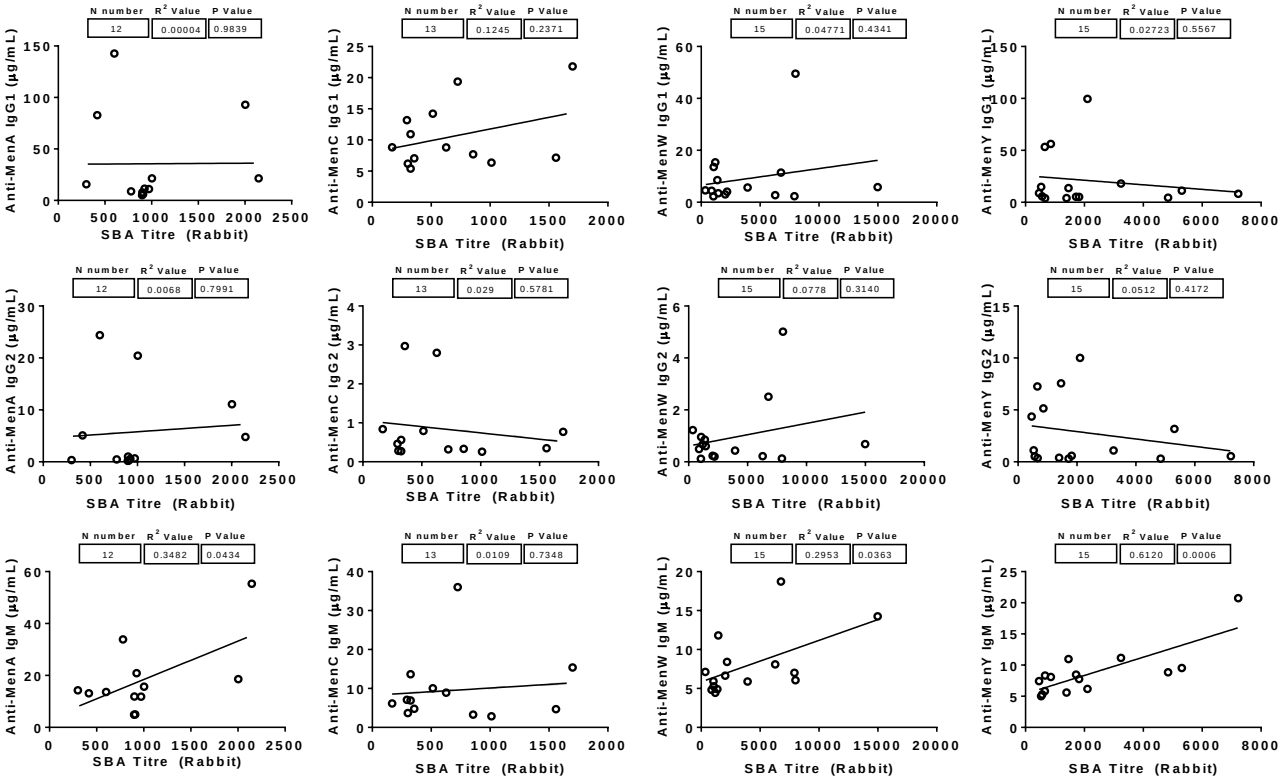


Figure 3. 14 Relationship between Antibody Subclass Response Post Vaccination with TT-Conjugated Quadrivalent Polysaccharide Vaccine and rSBA Titres

The concentration (µg/mL) of meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific IgG1, IgG2 and IgM antibody was measured in the serum of individuals four months post-vaccination with either one dose (n=23) or two doses of a TT-conjugated (n=26) quadrivalent meningococcal polysaccharide (serogroups A, C, W-135 and Y) vaccine (NB. The individuals shown here are the same as shown in ***Figure 3.11***, ***Figure 3.12*** and ***Figure 3.13***). Serum samples were taken as part of a clinical trial (identifier: NCT00718666) comparing the efficacy of one versus two doses of Nimenrix™ as described by Klein et al., (2013). Serum samples were taken and SBA titres (using rabbit serum as the source of complement; rSBA titre) were assessed to each serogroup as part of a clinical trial (identifier: NCT00718666) comparing the efficacy of one versus two doses of Nimenrix™ as described by Klein et al., (2013). Each scatter plot shows the relationship between the concentration of a serogroup-specific antibody subclass and hSBA titres four months post vaccination with one and two doses of the TT-conjugated quadrivalent polysaccharide vaccine (open circle; solid line). Each point represents the average serum concentration (µg/mL) of a serogroup-specific antibody subclass and the rSBA titre to the same serogroup of one individual. The number of samples in each group are indicated in each of the scatter plots. N numbers vary due to the availability of hSBA titres for each individual. The correlation (R value) is indicated for each group in each of the scatter plots which was calculated by linear regression. The statistical significance (P value) of each correlation is also shown.

3.6 Discussion

3.6.1 Antibody Response to Plain versus TT-Conjugated Quadrivalent Meningococcal Polysaccharide Vaccines

The concentration (μg/mL) of meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific IgG1, IgG2 and IgM antibody was measured in the serum of individuals one month post-vaccination with either a plain (Mencevax™) or a TT-conjugated (Nimenrix™) quadrivalent meningococcal polysaccharide (serogroups A, C, W-135 and Y) vaccine (***Figure 3. 7, Figure 3. 8*** and ***Table 3.3***). Serum samples were taken and SBA titres (using human serum as the source of complement; hSBA titre) were assessed to each serogroup as part of a clinical trial (identifier: NCT00427908) comparing the efficacy of both vaccines as described by Vesikaria et al., (2012).

A significantly higher concentration of polysaccharide-specific IgG1 and IgM antibodies was induced by TT-conjugated polysaccharides compared to plain polysaccharides for all serogroups (***Figure 3. 7***). Previous studies have shown that protein conjugation of meningococcal polysaccharides with TT produces significantly higher SBA titres when compared to plain polysaccharides by presumably inducing a stronger humoral immune response (Bermal et al., 2011; Dbaibo et al., 2012; Knuf et al., 2010; Lupisan et al., 2013; Memish et al., 2011; Pellegrino et al., 2015; Vesikari et al., 2012).

Protein conjugation of meningococcal polysaccharides (and other polysaccharide-based vaccines) aims to boost immunity to vaccination by altering the nature of B cell activation (Findlow et al., 2009; Lieberman et al., 1996; Sikkema et al., 2000). Bacterial polysaccharides induce T cell-independent B cell activation characterised by generally reduced antibody production along with minimal affinity maturation, class switching and memory compared to T cell-dependent B cell activation (Mond et al., 1995; Parker, 1993). Protein conjugation will drive antibody responses towards those seen with T cell-dependent B cell activation. This study has

confirmed that TT-conjugation of meningococcal polysaccharides gives higher SBA titres by inducing greater antibody responses.

The proportion of IgG1, IgG2, IgG3, IgG4 and IgM present in affinity-purified anti-MenACWY antibody isolated from the plasma of eleven adults was assessed by ELISA (***Figure 3. 1***). The predominant antibody subclass was IgG2 followed by IgM > IgG1 > IgG3 and IgG4. The individual antibody composition specific for each serogroup was not assessed. This predominant IgG2 antibody response measured in adults is dramatically different that measured in children where the IgG2 antibody response was very low (***Table 3.3*** and ***Table 3.4***). This difference in response to meningococcal polysaccharides may be explained by the difference in age of the individuals vaccinated. Previous studies have shown that for infants, children and adolescents the major subclass is IgG1 whereas a predominant IgG2 response is seen in adults (de Voer et al., 2011; Findlow et al., 2006; Holme et al., 2015; Joseph et al., 2004; Stoof et al., 2014).

3.6.2 Antibody Response to One versus Two Doses TT-Conjugated Quadrivalent Meningococcal Polysaccharide Vaccine

The concentration (µg/mL) of meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific IgG1, IgG2 and IgM antibody was measured in the serum of individuals four months post-vaccination with either one dose or two doses of TT-conjugated quadrivalent meningococcal polysaccharide (serogroups A, C, W-135 and Y) vaccine(***Table 3.4***, ***Figure 3. 11*** and ***Figure 3. 12***). Serum samples were taken as part of a clinical trial (identifier: NCT00718666) comparing the efficacy of one versus two doses of Nimenrix™ as described by Klein et al., (2013). The age of those vaccinated with one dose Nimenrix™ was 12 months. The age of those vaccinated with two doses Nimenrix™ was 9 months (first dose) and 12 months (second dose).

A significantly lower concentration of anti-MenA IgG1, IgG2, IgM and anti-MenC IgM antibody was measured four months post two doses TT-conjugated quadrivalent meningococcal vaccine compared to one dose (***Figure 3. 11***). In contrast, significantly higher titres of anti-MenW IgG2, anti-MenY IgG1 and IgG2 were seen after two doses TT-conjugated quadrivalent meningococcal vaccine compared to one dose. Hypo-responsiveness following vaccination with a TT-conjugated serogroup C polysaccharide vaccine has previously been described (Burrage et al., 2002). The exact mechanism behind this suppressed response to a second dose of the tetanus toxoid conjugated polysaccharide vaccine is unknown but may be as a result of CIES (Burrage et al., 2002). CEIS is thought to occur when antibodies specific to the conjugate protein sterically hinder B cell access to the polysaccharide antigen, when the immune response to the carrier protein is prioritised over the response to the polysaccharide antigen and/or when conjugate protein-specific regulatory T cells dampen the response to the polysaccharide antigen (Findlow and Borrow, 2016). Whether CEIS occurs following vaccination with a TT-conjugated quadrivalent meningococcal vaccine has not previously been described. The data from this study suggest a second dose of tetanus toxoid conjugated polysaccharide vaccine produces a suppressed response to serogroups A, C and W (***Figure 3. 11***). In contrast, a second dose of the vaccine appears to provide a boosted response to serogroup Y.

In contrast to antibody subclasses responses measured one month post vaccination, where IgM was the predominant response, the proportion of IgG1 and IgM antibodies four months post vaccination were not significantly different. This suggests that the concentration of IgM antibody drops more dramatically in the first four months after vaccination compared to the concentration of IgG1 antibody. However, it must be noted that the concentration of serogroup-specific antibodies measured one month post vaccination were in a cohort of individuals aged 2-10 years and the concentration of serogroup-specific antibodies measured

four months post vaccination were in a cohort of individuals aged 9-12 months and therefore may not be directly compared.

3.6.3 Relationship between Concentration of Antibody Subclasses and SBA Titres

The relationship between the concentration of serogroup-specific antibody subclasses and h/rSBA titres was investigated for each cohort (***Figure 3. 9***, ***Figure 3. 10***, ***Figure 3. 13*** and ***Figure 3. 14***). In cohorts vaccinated with the TT-conjugated quadrivalent meningococcal polysaccharide vaccine, hSBA titres correlated best with the concentration of serogroup-specific IgG1 antibody whereas rSBA titres correlated best with the concentration of serogroup-specific IgM antibody. The concentration of serogroup-specific IgG3 (and IgG4) antibody was not assessed in this study due to the low concentration of these subclasses in response to meningococcal vaccines and restricted volumes of sample available (***Figure 3. 1***). To fully appreciate the differential relationship between rSBA and hSBA titres with antibody responses to meningococcal vaccination IgG3 and IgG4 titres must be assessed. Despite its perceived low concentration in response to meningococcal polysaccharides, IgG3 most likely plays an important role in protection against invasive disease due to its enhanced complement fixing capabilities compared to the other human IgG subclasses.

A previous study, investigating the relationship between anti-MenC IgG and IgM and SBA titres in the presence of human and rabbit serum, showed a significant correlation between IgG and rSBA and hSBA titres (Santos et al., 2001). In the same study, anti-MenC IgM titres correlated well with rSBA titres but not with hSBA titre as shown in this study. Another study, showed significantly reduced survival of serogroup B coated with human IgM antibody in the presence of rabbit serum compared to human serum (Mandrell et al., 1995). As with these previous studies, the data from this study further suggest that serogroup-specific IgM

contributes significantly more to bactericidal titres in rSBAs compared to hSBAs and that the differential ability of human antibody subclasses to activate human and rabbit complement is most likely responsible for the poor correlation between rSBA and hSBA titres.

Chapter Four – Differences in Interaction between Human IgM and IgG Subclasses with Meningococcal Polysaccharides and Rabbit and Human C1q

4.1 Introduction

4.1.1 Interaction of Antibody Subclasses with Meningococcal Polysaccharides

The SBA measures the ability of vaccinee sera to kill *Neisseria meningitidis* bacteria (Goldschneider et al., 1969a; Maslanka et al., 1997). Bacteria are killed by mechanism of CDC driven by complement-activating antibody present in the sera of an individual acquired either naturally or by vaccination (Goldschneider et al., 1969a; Goldschneider et al., 1969b; Granoff et al., 1998).

It is thought that the affinity of antibody to bacteria significantly impacts upon SBA titres. Higher affinity antibodies are associated with higher SBA titres (Hetherington and Lepow, 1992; Schlesinger et al., 1992). One study, investigating the relationship between the concentration of meningococcal polysaccharide-specific IgG1 and IgG2 and antibody affinity to polysaccharide, showed that higher concentrations of IgG2 antibody were associated with a lower overall affinity of antibody (de Voer et al., 2011). In contrast, the concentration of IgG1 antibody significantly correlated with a higher overall affinity of antibody to meningococcal polysaccharide. These data suggest that the IgG1 antibody induced by vaccination with meningococcal polysaccharide are of a higher affinity than IgG2 antibody. However, the mechanisms responsible for the association of IgG1 antibody concentration and higher overall antibody affinity to meningococcal polysaccharide are not known. The affinity of individual antibody subclasses to polysaccharide has not been investigated.

4.1.2 Interaction of Human and Rabbit C1q with Antibody Subclasses

SBAs which measure the efficacy of new meningococcal vaccines utilise BRS as the source of complement. The relevance of BRS as the source of complement in SBAs in the licensure of new meningococcal vaccines has been questioned. Bactericidal titres in assays performed using BRS correlate poorly with the bactericidal titres in SBAs performed with human serum (Findlow et al., 2009; Gill et al., 2011a). In the previous chapter, I showed that hSBA titres correlated best with the concentration of serogroup-specific IgG1 antibody whereas rSBA titres correlated best with the concentration of serogroup-specific IgM antibody (***Chapter 3: Section 3.4.1*** and ***Section 3.5.1***). These data suggest that human IgG1 and IgM differentially activate human and rabbit complement.

Activation of the classical pathway of complement by antibody specific for the bacteria is the most important factor dictating the bactericidal activity of vaccinee sera in SBAs (Agarwal et al., 2014; Drogari-Apiranthitou et al., 2002; Goldschneider et al., 1969a; Granoff, 2009). The first component of the classical pathway, C1q, is activated by antigen-bound antibody (Cooper, 1985). The affinity of C1q to human antibody subclasses varies significantly. C1q exhibits the highest affinity to IgG3 antibody followed by IgG1, IgM, IgG2 and IgG4 (Bindon et al., 1988; Emanuel et al., 1982; Hughes-Jones, 1977; Moore et al., 2010; Painter et al., 1982; Patel et al., 2015; Quast et al., 2015). The affinity of rabbit C1q to human antibody subclasses has not been assessed. I hypothesise that the differential affinity of human and rabbit C1q to human antibody subclasses accounts for the poor correlation between hSBA and rSBA titres.

4.1.3 Chapter Aims

The aims of the work described in this chapter are as follows:

i. To investigate functional and kinetic differences between the interaction of human antibody subclasses with meningococcal polysaccharide vaccine antigens.

ii. To establish and quantitate any differences between the interaction of rabbit and human C1q with human IgM and IgG subclasses.

4.2 Competitive ELISA

It is thought that the affinity of antibody to bacteria significantly impacts upon SBA titres with higher affinity antibodies being associated with higher SBA titres (Hetherington and Lepow, 1992; Schlesinger et al., 1992). The affinity of individual antibody subclasses to polysaccharide has not been investigated but it is hypothesised that certain subclasses are of a higher affinity and therefore contribute more to SBA titres. To address this the affinity of IgG1, IgG2, IgG3 and IgG4 and IgM antibody subclasses to meningococcal polysaccharide (serogroups A, C, W-135 and Y) was assessed by competitive ELISA.

The competitive ELISA measures the affinity of an antibody to antigen by measuring the concentration of fluid-phase antigen (mixed with the antibody) required to block antibody binding to solid-phase antigen (adsorbed onto an ELISA plate). The concentration of fluid-phase antigen blocking 50% binding to solid-phase antigen is considered the 'functional affinity' of the antibody to the antigen.

4.2.1 Interaction of Antibody Subclasses with Meningococcal Polysaccharides

In preparation for the meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific IgG1, IgG2, IgG3 and IgG4 and IgM competitive ELISA assays, the dilution factor required in each competitive ELISA assay for each plasma sample (n=14) was assessed by ELISA (***Figure 4.1***). In this way, the ELISA signal achieved with each plasma sample in each competitive ELISA assay was normalised. Plasma samples were taken from adult individuals at least one month post-vaccination with a quadrivalent plain polysaccharide (serogroups A, C, W-135 and Y) meningococcal vaccine (Mencevax™). Plasma samples were diluted in duplicate across ELISA plates coated with a mixture of TT-conjugated meningococcal polysaccharides from serogroups A, C, W and Y. Subsequent IgG1, IgG2, IgG3, IgG4 or IgM antibody binding was detected with either a rabbit anti-human IgG1, a rabbit anti-human IgG2, a rabbit anti-human IgG3, a rabbit anti-human IgG4 or a donkey anti-human IgM antibody (Stratech). Typical binding curves for each antibody subclass selected from five representative individuals are shown in ***Figure 4.1a***. The dilution factors required for each plasma sample with each antibody subclass to achieve an absorbance of 1.0 (492nm) are shown in ***Figure 4.1b***.

The functional affinity of IgG1 (n=12), IgG2 (n=10), IgG3 (n=6), IgG4 (n=6) and IgM (n=12) antibody to TT-conjugated meningococcal polysaccharides in vaccinee plasma of these fourteen adult individuals to the meningococcal polysaccharide (serogroups A, C, W-135 and Y) was then assessed by competitive ELISA (***Figure 4.2a*** and ***Figure 4.2c***). The number of individuals varies between antibody subclasses due to the sample exclusion criteria described in ***Figure 4.1*** and ***Figure 4.3***. Vaccinee plasma was diluted to the factor calculated in ***Figure 4.1*** and incubated with a range of concentrations of fluid-phase TT-MenACWY. Plasma samples were then incubated on plates coated with TT-conjugated meningococcal polysaccharides and binding of IgG1, IgG2, IgG3, IgG4 and IgM measured at each concentration of fluid-phase TT-MenACWY. Representative curves showing inhibition of binding of IgG1, IgG2, IgG3, IgG4 and IgM to TT-

MenACWY-coated plates at each concentration of fluid-phase TT-MenACWY are shown in ***Figure 4.2b***. The concentration of TT-conjugated meningococcal polysaccharides which inhibited 50% binding of the specific antibody subclass was calculated as the functional affinity. The geometric mean of the functional affinity for antibody subclasses for TT-conjugated meningococcal polysaccharides was calculated as $3.0x10^{-10}$M (IgG1; n=12), $1.3x10^{-9}$M (IgG2; n=10), $4.4x10^{-10}$M (IgG3; n=6), $3.7x10^{-9}$M (IgG4; n=6) and $3.7x10^{-10}$M (IgM; n=12) (***Figure 4. 2c***).

Plasma samples were diluted as described in ***Figure 4.1*** and incubated with a range of concentrations ($1x10^{-7}$ to $4x10^{-13}$M) of tetanus toxoid-conjugated meningococcal polysaccharide from serogroups A, C, W-135 and Y (TT-MenACWY). Each plasma:TT-MenACWY mixture, plasma control only or buffer only control (blank) was then incubated in triplicate on ELISA plates coated with TT-MenACWY. Each of the plasma:TT-MenACWY mixtures were aspirated and incubated in duplicate on a second set of ELISA plates coated with TT-MenACWY. Subsequent IgG1, IgG2, IgG3, IgG4 or IgM antibody binding (492nm) was detected on both sets of ELISA plates. The correlation between the absorbance values achieved on the first set and second set of TT-MenACWY ELISA plates to assess whether the binding equilibrium between antibody subclasses to TT-MenACWY had not been significantly disturbed after incubation on the first set of TT-MenACWY ELISA plates. (***Figure 4. 3***). The mean R-squared values for each antibody subclass was calculated as 0.98±0.02 (IgG1), 0.99±0.01 (IgG2), 0.97±0.03 (IgG3), 0.97±0.02 (IgG4) and 0.97±0.03 (IgM). Samples with R-squared values <0.9 (binding equilibrium disturbed by more than 10%) were excluded from analysis. In total, three samples were excluded.

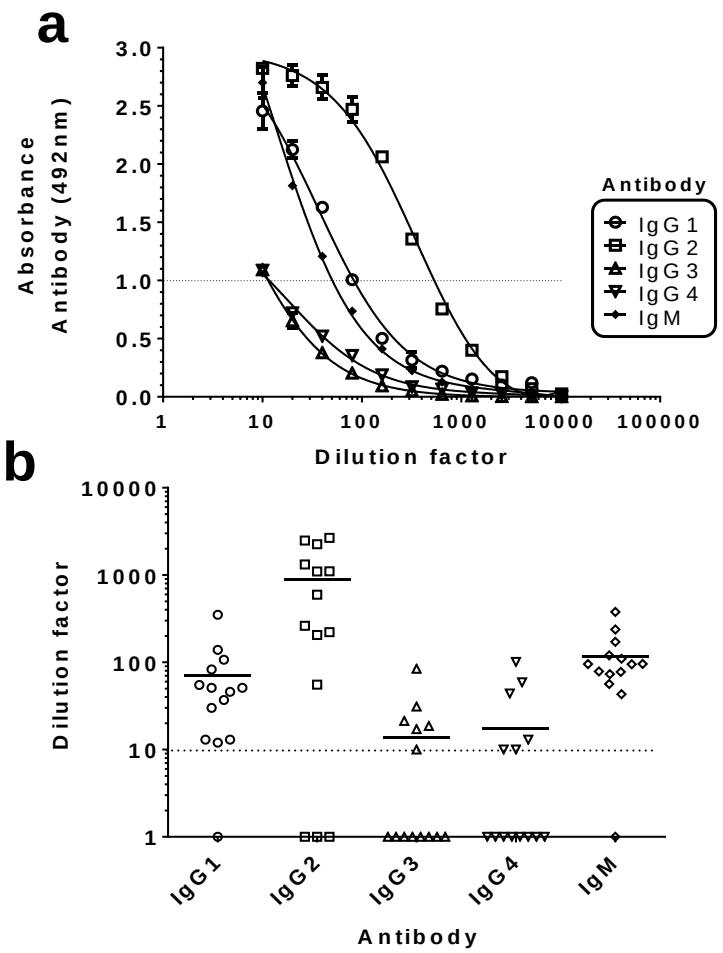
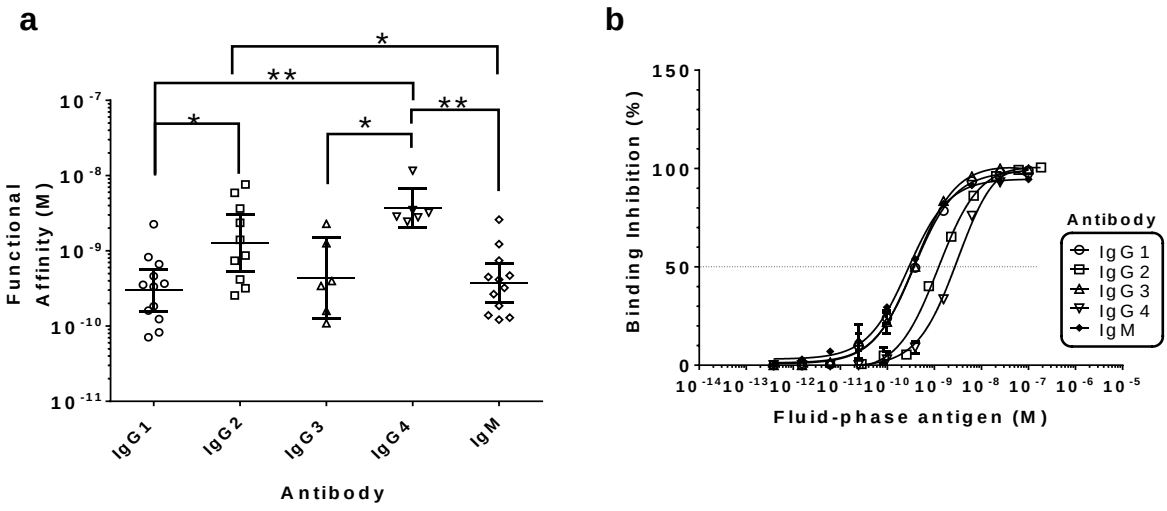


Figure 4. 1 Sample Dilution Factor Calculations for Competitive ELISA Assay

In preparation for the meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific IgG1, IgG2, IgG3 and IgG4 and IgM competitive ELISA assays, the dilution factor required in each competitive ELISA assay for each plasma sample (n=14) was assessed by ELISA. In this way, the ELISA signal achieved with each plasma sample in each competitive ELISA assay was normalised. Plasma samples were taken from individuals at least one month post-vaccination with a quadrivalent plain polysaccharide (serogroups A, C, W-135 and Y) meningococcal vaccine (Mencevax™). Plasma samples were diluted in duplicate across ELISA plates coated with a mixture of TT-conjugated meningococcal polysaccharides from serogroups A, C, W and Y. Subsequent IgG1, IgG2, IgG3, IgG4 or IgM antibody binding was detected with either a rabbit anti-human IgG1, a rabbit anti-human IgG2, a rabbit anti-human IgG3, a rabbit anti-human IgG4 or a donkey anti-human IgM antibody (Stratech). ***a***, Typical binding curves for each antibody subclass selected from five representative individuals. Each point represents the average absorbance achieved at each plasma sample dilution factor for each subclass. The errors bars represent the standard deviation. The solid line represents the standard curve model (non-linear regression, sigmoidal, 4PL standard curve) used to interpolate the dilution factor of the plasma sample required to achieve an absorbance of 1.0 (492nm; dotted line) for each antibody subclass. ***b***, The dilution factors required for each plasma sample with each antibody subclass to achieve an absorbance of 1.0 (492nm). Each point represents the average dilution factor of the plasma from one individual for each competitive ELISA assay. Samples achieving an absorbance lower than 1.0 (492nm) at a dilution factor of 1/10 (dotted line) were assigned a value of 1 and omitted from the competitive ELISA due to restraints on sample volume. The horizontal line represents the mean average dilution factor by antibody subclass.



c

Functional Affinity (M)					
Antibody	*IgG1*	*IgG2*	*IgG3*	*IgG4*	*IgM*
Geometric Mean	3.0E-10	1.3E-09	4.4E-10	3.7E-09	3.7E-10
Lower 95% CI	1.6E-10	5.4E-10	1.3E-10	2.0E-09	2.1E-10
Upper 95% CI	5.7E-10	3.1E-09	1.5E-09	6.7E-09	6.8E-10

Figure 4. 2 Functional Affinity of Antibody Subclasses to Meningococcal Polysaccharides

The functional affinity (M) of meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific IgG1, IgG2, IgG3 and IgG4 and IgM antibodies present in the plasma of fourteen individuals to the meningococcal polysaccharide (serogroups A, C, W-135 and Y) was assessed by competitive ELISA as described in ***Section 2.5.1.1***. ***a***, The functional affinity of IgG1 (n=12), IgG2 (n=10), IgG3 (n=6), IgG4 (n=6) and IgM (n=12) antibody to TT-conjugated meningococcal polysaccharides in vaccinee plasma. Each point represents the functional affinity of antibody subclasses to TT-MenACWY for one individuals. The errors bars represent the geometric mean (middle line) with the 95% confidence intervals (top and bottom lines) of an antibody subclass. Significant differences between the functional affinity of antibody subclasses were compared by t-test analysis. The asterisks indicate the statistical significance of the difference between the functional affinity of antibody subclasses (* = P<0.05; ** = P value <0.01). N numbers vary due the exclusion factors of samples as described in ***Figure 4.1*** and ***Figure 4.3***. ***b***, Representative curves showing inhibition of binding of IgG1, IgG2, IgG3, IgG4 and IgM to TT-MenACWY-coated plates following incubation of antibody with a range of concentrations of fluid-phase TT-MenACWY. Each point represents the average inhibition of antibody binding to the TT-MenACWY ELISA plate at each concentration of TT-MenACWY incubated with the plasma sample. Error bars represent the standard deviation. The solid lines represent the standard curve model used to interpolate the concentration of TT-MenACWY resulting in 50% binding inhibition to the TT-MenACWY-coated ELISA plate (non-linear regression; sigmoidal, 4PL standard curve). The dotted line shows the concentration of 50% inhibition of antibody subclass binding to plates was calculated as the functional affinity of antibody (dotted line). ***c***, Table summarising the geometric mean and the 95% confidence intervals of the functional affinities of each antibody subclasses to TT-MenACWY.

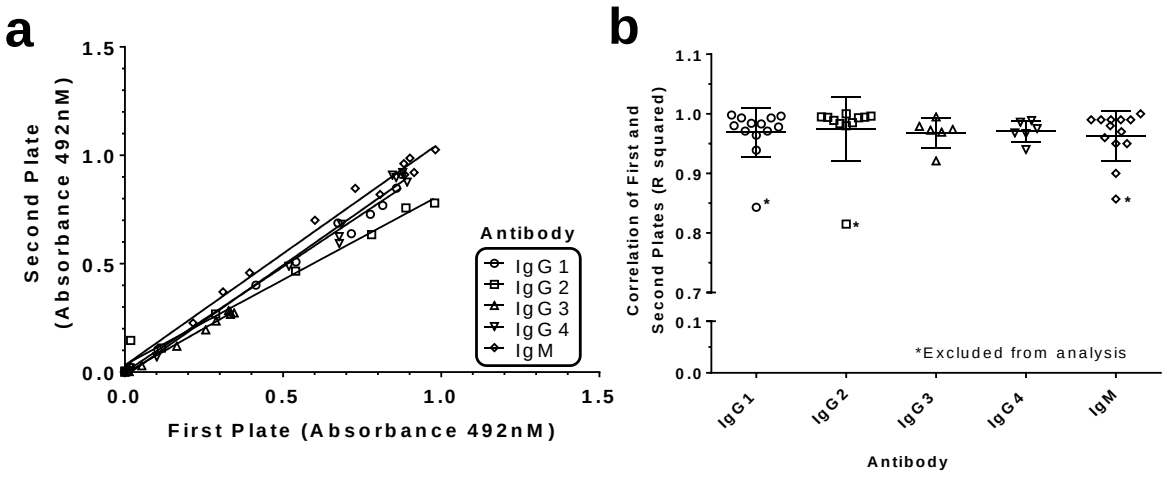


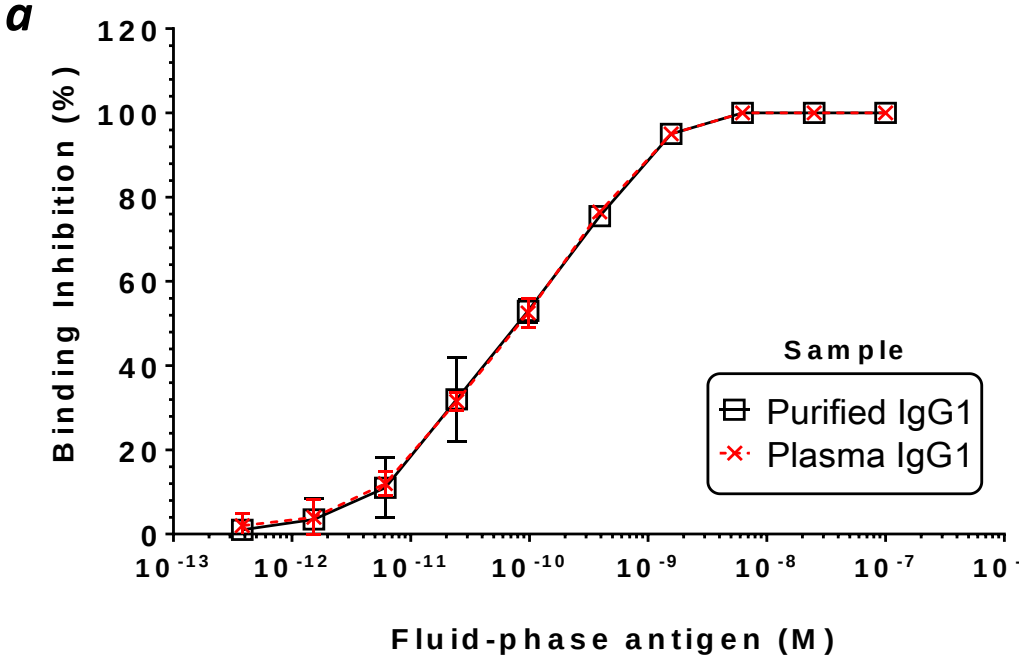
Figure 4. 3 Competitive ELISA: Analysis of First and Second Plates

The functional affinity (M) of meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific IgG1, IgG2, IgG3 and IgG4 and IgM antibodies present in the plasma of fourteen individuals to the meningococcal polysaccharide (serogroups A, C, W-135 and Y) was assessed by competitive ELISA as described in ***Figure 4.2***. Plasma samples were diluted as described in ***Figure 4.1*** and incubated with a range of concentrations ($1x10^{-7}$ to $4x10^{-13}$M) of tetanus toxoid-conjugated meningococcal polysaccharide from serogroups A, C, W-135 and Y (TT-MenACWY). Each plasma:TT-MenACWY mixture, plasma control only or buffer only control (blank) was then incubated in triplicate on ELISA plates coated with TT-MenACWY. Each of the plasma:TT-MenACWY mixtures were aspirated and incubated in duplicate on a second set of ELISA plates coated with TT-MenACWY. Subsequent IgG1, IgG2, IgG3, IgG4 or IgM antibody binding (492nm) was detected on both sets of ELISA plates. The correlation between the absorbance values achieved on the first set and second set of TT-MenACWY ELISA plates to assess whether the binding equilibrium between antibody subclasses to TT-MenACWY had not been significantly disturbed after incubation on the first set of TT-MenACWY ELISA plates. ***a***, Representative correlations between the absorbance achieved on the first and second set of ELISA plates at each concentration of TT-MenACWY incubated with plasma samples. Each point represents the absorbance achieved on the first and second set of ELISA plates at each concentration of TT-MenACWY incubated with plasma samples for IgG1, IgG2, IgG3, IgG4 and IgM antibodies. Solid lines represent the model of linear regression fit to each data used to calculate the R-squared values for each antibody subclass in each sample. ***b***, The correlation (R-squared values) of the absorbance achieved on the first and second set of ELISA plates for IgG1 (n=12), IgG2 (n=11), IgG3 (n=6), IgG4 (n=6) and IgM (n=13) antibodies. N numbers vary due the exclusion factors of samples as described in ***Figure 4.1***. Each point represents the R-squared calculated for each antibody subclasses for one individual. The errors bars represent the geometric mean (middle line) with the 95% confidence intervals (top and bottom lines) of R-values for each subclass.). R-squared values lower than 0.9 suggests a greater than 10% disturbance in binding equilibrium between antibody subclasses to TT-MenACWY after incubation on the first on TT-MenACWY-coated ELISA plate. Samples with R-squared < 0.9 were excluded from analysis (asterisked). Three samples were excluded in total.

4.2.2 Functional Affinity of Purified and Plasma IgG1 to Meningococcal Polysaccharides

The functional affinities of antibodies of different subclasses were assessed in plasma by competitive ELISA. However, other antibody subclasses were present in plasma which may impact accurate quantitation of functional affinity by competing for binding and thus interfering with absolute antibody concentration. To address this, the functional affinities of IgG1 in a plasma sample (pool of 14 individuals previously vaccinated with Mencevax™) and purified non-specific IgG1 (from the same pooled sample) to TT-MenACWY were compared (***Figure 4. 4***). The functional affinity of plasma IgG1 was calculated as 8.7×10^{-11}M and the functional affinity of purified IgG1 was calculated as 8.4×10^{-11}M. By one-way ANOVA statistical analysis, there was no significant difference between the functional affinities of plasma IgG1 and purified IgG1 antibodies to TT-conjugated meningococcal polysaccharides. These data concluded that the other antibody subclasses present in plasma in addition to the antibody subclass being assessed do not impact on the functional affinity of the antibody subclass.

a



b

Sample	*Functional Affinity (M)*	*Standard Deviation*
Plasma IgG1	8.725E-11	1.212E-11
Purified IgG1	8.351E-11	1.527E-11

Figure 4. 4 Functional Affinity of Purified and Plasma IgG1 to Meningococcal Polysaccharides

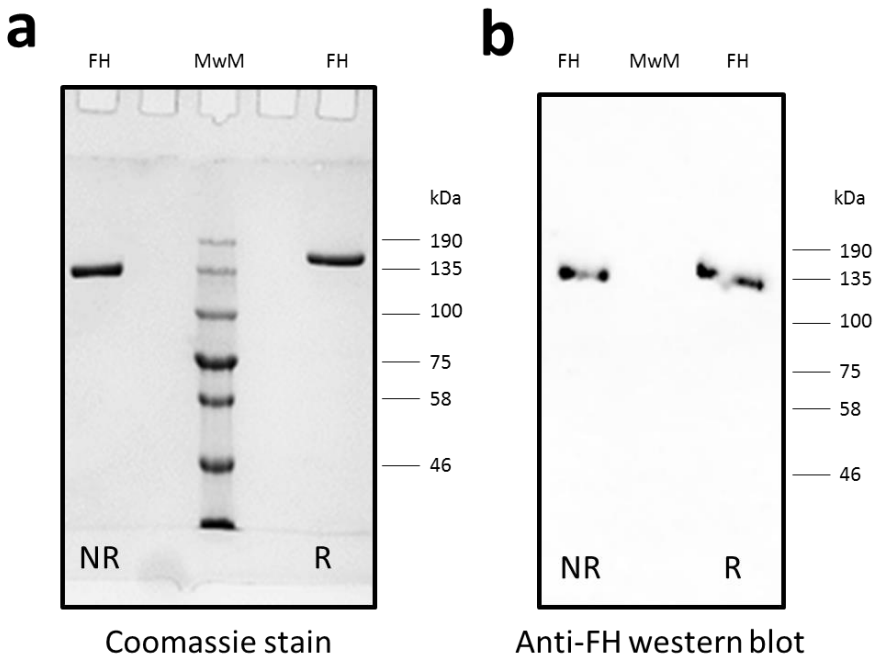
The functional affinity (M) of meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific IgG1, IgG2, IgG3 and IgG4 and IgM antibodies present in the plasma of fourteen individuals to the meningococcal polysaccharide (serogroups A, C, W-135 and Y) was assessed by competitive ELISA as described in ***Figure 4.2***. In the competitive ELISA, other antibody subclasses are present in plasma in addition to the antibody subclass being assessed which may impact accurate quantitation of functional affinity by competing for binding and thus interfering with absolute antibody concentration. To address this, the functional affinities of IgG1 in a plasma sample (pool of 14 individuals previously vaccinated with Mencevax™) and purified non-specific IgG1 (from the same pooled sample) to TT-MenACWY were compared. ***a***, Plasma or purified non-specific IgG1 were incubated with a range of concentrations TT-MenACWY. Each sample:TT-MenACWY mixture was then incubated in triplicate on ELISA plates coated with TT-MenACWY. Subsequent IgG1 antibody binding (492nm) was detected. The concentration (M) of TT-MenACWY inhibiting 50% IgG1 binding was interpolated by fitting the data with a non-linear regression; sigmoidal, 4PL standard curve. Each point represents the average inhibition of IgG1 binding to the TT-MenACWY ELISA plate at each concentration of TT-MenACWY incubated with plasma IgG1 (red crosses) or purified IgG1 (open squares). Error bars represent the standard deviation. ***b***, Summary table of the functional affinities (M) and standard deviations of plasma IgG1 and purified IgG1 antibodies to TT-MenACWY. By one-way ANOVA statistical analysis, there was no significant difference between the functional affinities of plasma IgG1 and purified IgG1 antibodies to TT-conjugated meningococcal polysaccharides showing that the other antibody subclasses present in plasma in addition to the antibody subclass being assessed do not impact on the functional affinity of the antibody subclass.

4.2.3 Functional Affinity and KD of Two Anti-FH Antibodies

To validate the use of this assay, the functional affinity of two anti-human FH antibodies (OX-24 and MBI-7) to human FH (variant H402) was measured by competitive ELISA (***Figure 4. 5***); the KD of both antibodies had been determined previously using SPR. Human FH (variant H402) was purified from human plasma by affinity chromatography and competitive ELISA run as before. The functional affinity of antibody OX-24 was calculated as 2.2×10^{-10}M and the functional affinity of antibody MBI-7 was calculated as 1.1×10^{-9}M.

4.3 Interaction of Human and Rabbit C1q with Antibody Subclasses

There is evidence that human antibody subclasses differentially activate human and rabbit complement. It is hypothesised that this contributes to the poor correlation between SBA titres when either human or rabbit serum is used as the source of complement (Findlow et al., 2009; Gill et al., 2011a; Griffiss and Goroff, 1983; Mandrell et al., 1995; Santos et al., 2001; Zollinger and Mandrell, 1983). To test this hypothesis, the interaction of human and rabbit C1q to different antibody subclasses was investigated by ELISA and SPR. In preparation for these assays, human antibody subclasses and human and rabbit C1q were purified as described below.



c

Antibody	Method	
	Surface Plasmon Resonance (KD)	***Competitive ELISA (Functional Affinity)***
OX-24	$1.2x10^{-10}$ M	$2.2x10^{-10}$M
MBI-7	$2.0x10^{-9}$ M	$1.1x10^{-9}$M

Figure 4. 5 Functional Affinity and KD of Two Anti-FH Antibodies

The functional affinity (M) of meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific IgG1, IgG2, IgG3 and IgG4 and IgM antibodies present in the plasma of fourteen individuals to the meningococcal polysaccharide (serogroups A, C, W-135 and Y) was assessed by competitive ELISA as described in ***Figure 4.2***. To validate the use of the competitive ELISA assay, the functional affinity of two anti-human FH antibodies (OX-24 and MBI-7) to human FH (variant H402), which had been previously assessed by surface plasmon resonance, was measured by competitive ELISA. ***a***, Firstly, human FH was affinity purified for use in the completive ELISA as described in ***Section 2.2.7***. The purity the purified human FH was assessed by SDS-PAGE. 200ng of the purified antibody was run through a 9% polyacrylamide gel under non-reducing (NR) and reducing conditions (R) and stained with coomassie Brilliant Blue dye. Under non-reducing (NR) conditions FH appeared as a single band of 146.6kDa. Under reduced conditions (R) FH appeared as a single band of 150.4kDa. No contaminating proteins were detected in the human FH preparation. ***b***, By western blot analysis probing with a mouse anti-human FH antibody (OX-24), the 146.6kDa band (NR) and the 150.4kDa band (R) were confirmed as FH. ***c***, A summary table detailing the KD and functional affinity of OX-24 and MBI-7 antibodies to human FH as measured by surface plasmon resonance and competitive ELISA, respectively.

4.3.1 Isolation of Antibody Subclasses

Non-specific human IgG1, IgG2, IgG3, IgG4, IgM and non-specific rabbit IgG antibodies were purified to homogeneity by affinity chromatography. To assess purity and calculate protein mass, each antibody preparation was run through a 9% polyacrylamide gel under reducing conditions and stained with coomassie Brilliant Blue dye (***Figure 4. 6***). Using the molecular weight marker (MwM) as the standard, the molecular weights (kDa) of each antibody heavy chain (HC) were interpolated as described in ***Chapter 2: Section 2.4.1.1***. The molecular weights of rabbit IgG HC was calculated as 51.7kDa, human IgG1 HC as 51.0kDa, IgG2 HC as 49.4kDa, IgG3 HC as 61.1kDa, IgG4 HC as 51.5kDa and IgM HC as 86.9kDa. By western blot, no contamination of the purified non-specific human IgG subclasses with other IgG subclasses could be detected *(**Figure 4. 7***).

Human anti-meningococcal polysaccharide (serogroups A, C, W and Y) antibody (anti-MenACWY) and human IgG (non-specific) antibody were affinity purified from plasma using meningococcal polysaccharide-conjugated Sepharose or Protein G, respectively, and used in the C1q-binding assay. The human anti-meningococcal polysaccharide (serogroups A, C, W and Y) antibody was purified from the plasma of 14 adult individuals taken at least one month post vaccination with a plain meningococcal polysaccharide (serogroups A, C, W and Y) vaccine (Mencevax™). The non-specific human IgG was purified from human plasma taken from three individuals. The antibody composition of both antibody preparations was measured by ELISA. The composition of the non-specific IgG purified from human plasma was calculated as 72.3% IgG1, 16.2% IgG2, 8.5% IgG3 and 3.1% is IgG4 (***Figure 4. 8***). The antibody subclass composition of the anti-MenACWY antibody was 23.4% IgG1, 37.3% IgG2, 4.0% IgG3, 3.1% IgG4 and 30.0% IgM (***Figure 4. 8***).

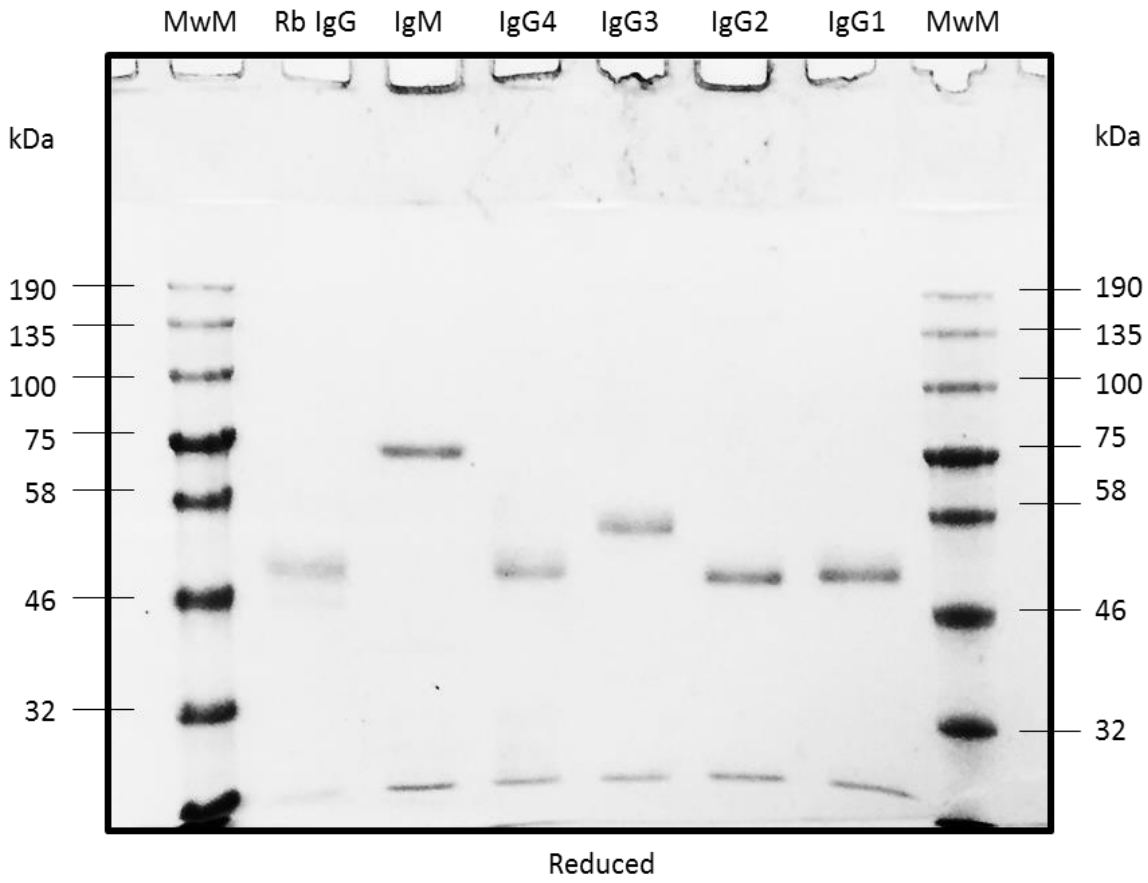


Figure 4. 6 Coomassie Stain of Human IgM, IgG1, IgG2, IgG3, IgG4 and Rabbit IgG Antibodies

Human IgM, IgG1, IgG2, IgG3, IgG4 and rabbit IgG antibodies were affinity purified from plasma. The purity and composition of each antibody preparation was assessed by SDS-PAGE. 200ng of each antibody preparation was run though a 9% polyacrylamide gel under reducing conditions and stained with coomassie Brilliant Blue dye. Molecular weight markers (MwM) were run either side of the gel and used to interpolate the molecular weight (kDa) of each protein band. The molecular weight of the heavy chain of each antibody preparation was calculated as 51.7kDa (rabbit IgG), 51.0kDa (human IgG1), 49.4kDa (human IgG2), 61.1kDa (human IgG3), 51.5kDa (human IgG4) and 86.9kDa (human IgM). No contaminating proteins could be detected in any antibody preparation.

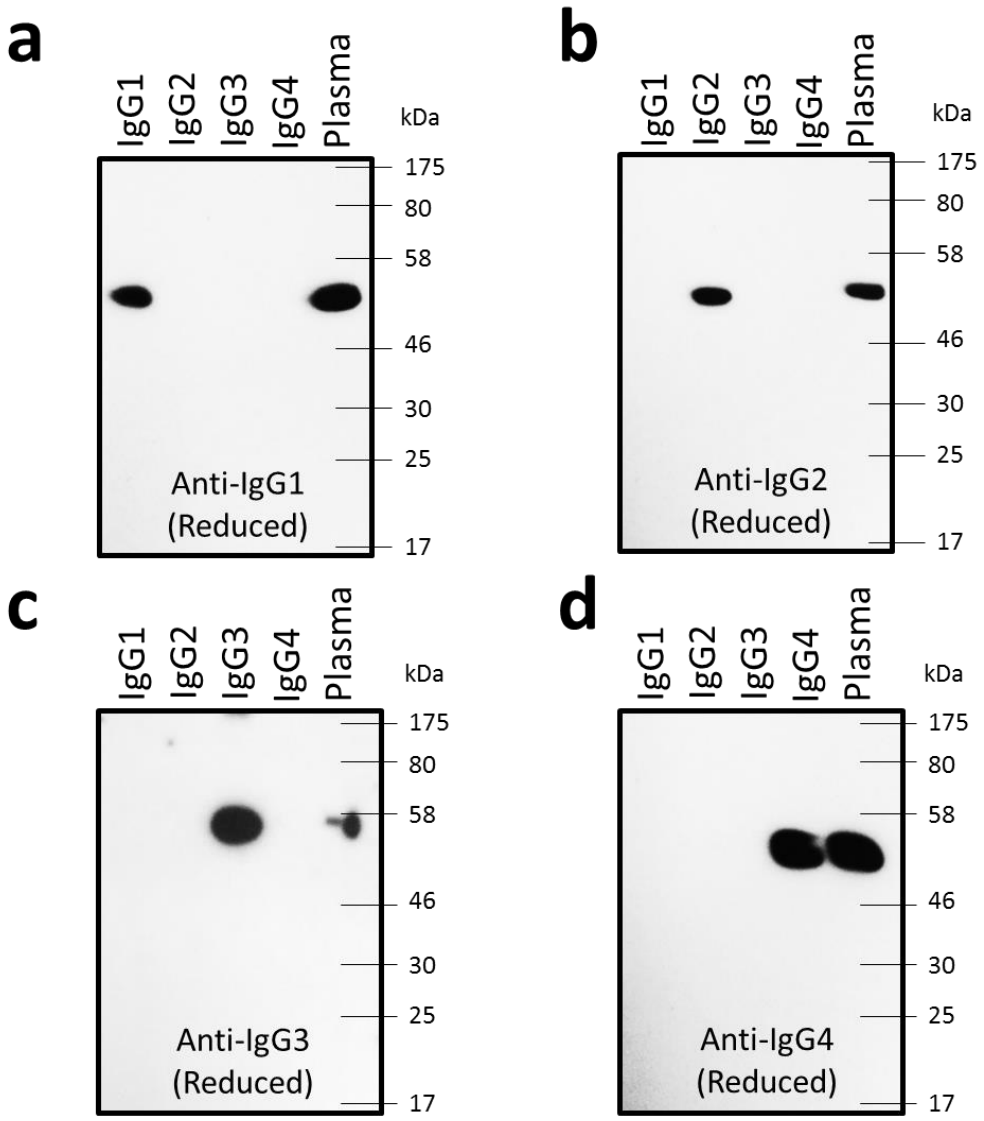


Figure 4. 7 Western Blot Analysis of Human IgG1, IgG2, IgG3 and IgG4 Antibody Preparations

Human IgG1, IgG2, IgG3 and IgG4 antibodies were affinity purified from plasma. By western blot, each IgG subclass preparation was assessed for contamination with any other of the IgG subclasses. 200ng of each antibody preparation was run through four 12.5% polyacrylamide gels under reducing conditions. 10µL human plasma (diluted 1 in 500) was also run on each gel as a positive control for each antibody subclass. Once run, gels were transferred onto nitrocellulose membrane and probed with either a rabbit anti-human IgG1 (blot ***a***), a rabbit anti-human IgG2 (blot ***b***), a rabbit anti-human IgG3 (blot ***c***) or a rabbit anti-human IgG4 (blot ***d***) antibody (Stratech). In blot ***a*** (anti-human IgG1), positive bands were detected in the lanes containing purified human IgG1 and plasma only. In blot ***b*** (anti-human IgG2), positive bands were detected in the lanes containing purified human IgG2 and plasma only. In blot ***c*** (anti-human IgG3), positive bands were detected in the lanes containing purified human IgG3 and plasma only. In blot ***d*** (anti-human IgG4), positive bands were detected in the lanes containing purified human IgG4 and plasma only. These data confirmed that there was no detectable contamination of each IgG subclass preparation with any other of the IgG subclasses.

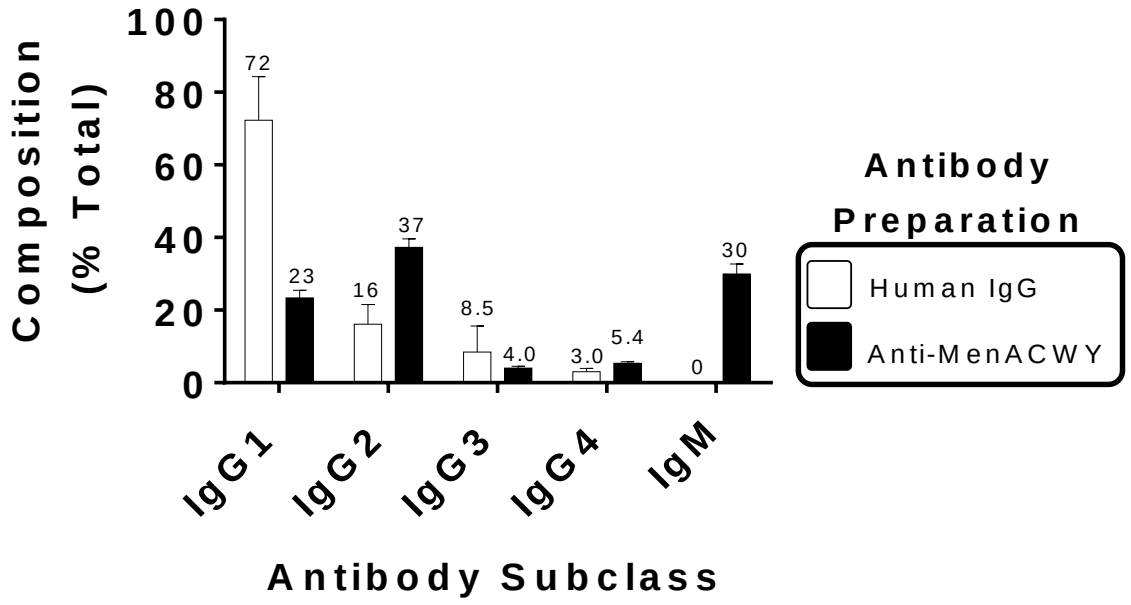


Figure 4. 8 Antibody Subclass Composition of Human IgG and Anti-MenACWY Antibody Preparations

Meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific antibody was affinity-purified from the plasma of fourteen adult individuals. Plasma samples were taken at least one month post-vaccination with a quadrivalent plain polysaccharide (serogroups A, C, W-135 and Y) meningococcal vaccine (Mencevax™). Non-specific human IgG was affinity-purified from the plasma of three adult individuals using a protein G-conjugated Sepharose column. The concentration of IgG1, IgG2, IgG3, IgG4 and IgM present in the antibody purified from each individual was assessed by running each sample (in duplicate) though a commercial IgM ELISA kit and a commercial IgG subclass ELISA kit. The proportion of each antibody subclass in the meningococcal polysaccharide specific antibody (solid bars) and purified human IgG (open bars) was calculated as the percentage of total antibody concentration (e.g. the concentration of IgG1 antibody present in the sample over the concentration of IgG1 plus IgG2 plus IgG3 plus IgG4 plus IgM present in the sample). The mean proportion of each antibody subclass present in the purified meningococcal polysaccharide-specific antibody was calculated as 23% IgG1, 37% IgG2, 4.0% IgG3, 3.0% IgG4 and 30% IgM. The mean proportion of each antibody subclass present in the purified human IgG antibody was calculated as 72% IgG1, 16% IgG2, 8.5% IgG3, 5.4% IgG4 and 0.0% IgM. Each point represents the average proportion of an antibody subclass present in the antibody purified from one individual. The errors bars represent the mean proportion (middle line) with the standard deviation (top and bottom lines) of an antibody subclass.

4.3.2 Human and Rabbit C1q Binding ELISA

To investigate the differences in the interaction of human and rabbit C1q to different antibody subclasses, binding of human and rabbit C1q to solid-phase human IgG1, IgG2, IgG3, IgG4, non-specific IgG, meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific antibody and rabbit IgG was measured by ELISA (***Figure 4.9***). Human serum (4%; ***Figure 4.9a***), rabbit serum (4%; ***Figure4.9b***) or buffer only (no serum control) was incubated in duplicate on either blank ELISA wells or ELISA wells coated with 30µg/mL of each antibody subclass preparation. Subsequent human and rabbit C1q binding to each antibody subclass was detected with a sheep anti-human C1q antibody. The absorbance achieved following incubation of serum or buffer only (no serum) with each antibody preparation was compared by t-test statistical analysis. Significant human C1q binding was detect with human IgG3, human IgG1, human IgG, anti-MenACWY and rabbit IgG. Significant rabbit C1q binding was detect with human IgG3, human IgG1, human IgG and human IgM. These data highlight important differences in the ability of human and rabbit C1q to bind human antibody subclasses.

4.3.3 Isolation of Human and Rabbit C1q

In preparation for the SPR assays, human and rabbit C1q was purified to homogeneity from serum by cation exchange chromatography and affinity chromatography on natural ligand (crosslinked Ig), respectively. The purity and composition of human and rabbit C1q was assessed by SDS-PAGE (***Figure 4. 10***).). As well as C1q, other protein bands (assumed to aggregates of C1q and other contaminants) were also detected in both preparations concluding that further purification of both human and rabbit C1q was necessary. Contaminating proteins, aggregates and fragments were removed from each preparation by injection over a 24ml Superdex 75 size exclusion chromatography column (***Figure 4. 11***).

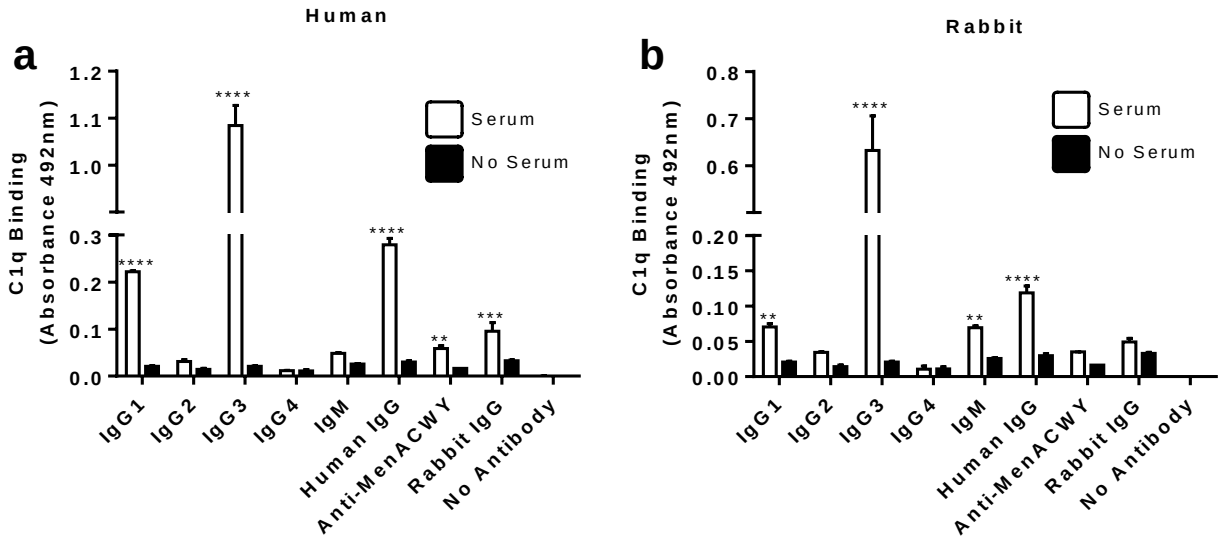


Figure 4. 9 Human and Rabbit C1q Antibody Binding ELISA

To investigate the differences in the interaction of human and rabbit C1q to different antibody subclasses, binding of human and rabbit C1q to solid-phase human IgG1, IgG2, IgG3, IgG4, non-specific IgG, meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific antibody and rabbit IgG was measured by ELISA. Human IgG1, IgG2, IgG3, IgG4, IgM, non-specific IgG and rabbit IgG antibodies were purified from plasma by affinity chromatography (as detailed in ***Section 2.2.2*** and ***Section 2.2.4***). The meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific antibody was affinity-purified from the pooled plasma of fourteen adult individuals taken at least one month post-vaccination with a quadrivalent plain polysaccharide (serogroups A, C, W-135 and Y) meningococcal vaccine (as detailed in ***Section 2.2.3***). The antibody subclass compositions of the non-specific human IgG and meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific antibody preparation are detailed in ***Figure 4.8***. Human serum (4%; ***a***), rabbit serum (4%; ***b***) or buffer only (***a*** and ***b***; no serum control) was incubated in duplicate on either blank ELISA wells or ELISA wells coated with 30µg/mL of each antibody subclass preparation. Subsequent human and rabbit C1q binding to each antibody subclass was detected with a sheep anti-human C1q antibody. Each bar represents the mean average absorbance achieved following incubation of either serum (open bars) or buffer only (solid bars) with each antibody preparation. The error bars represent the standard deviation. The absorbance achieved following incubation of serum or buffer only (no serum) with each antibody preparation was compared by t-test statistical analysis. The asterisks indicate the statistical significance of the difference between the average absorbance achieved with serum and buffer alone by antibody subclass (**** = P value<0.0001; ** = P value<0.01). ***a***, Significant human C1q binding was detect with human IgG3, human IgG1, human IgG, anti-MenACWY and rabbit IgG. ***b***, Significant rabbit C1q binding was detect with human IgG3, human IgG1, human IgG and human IgM.

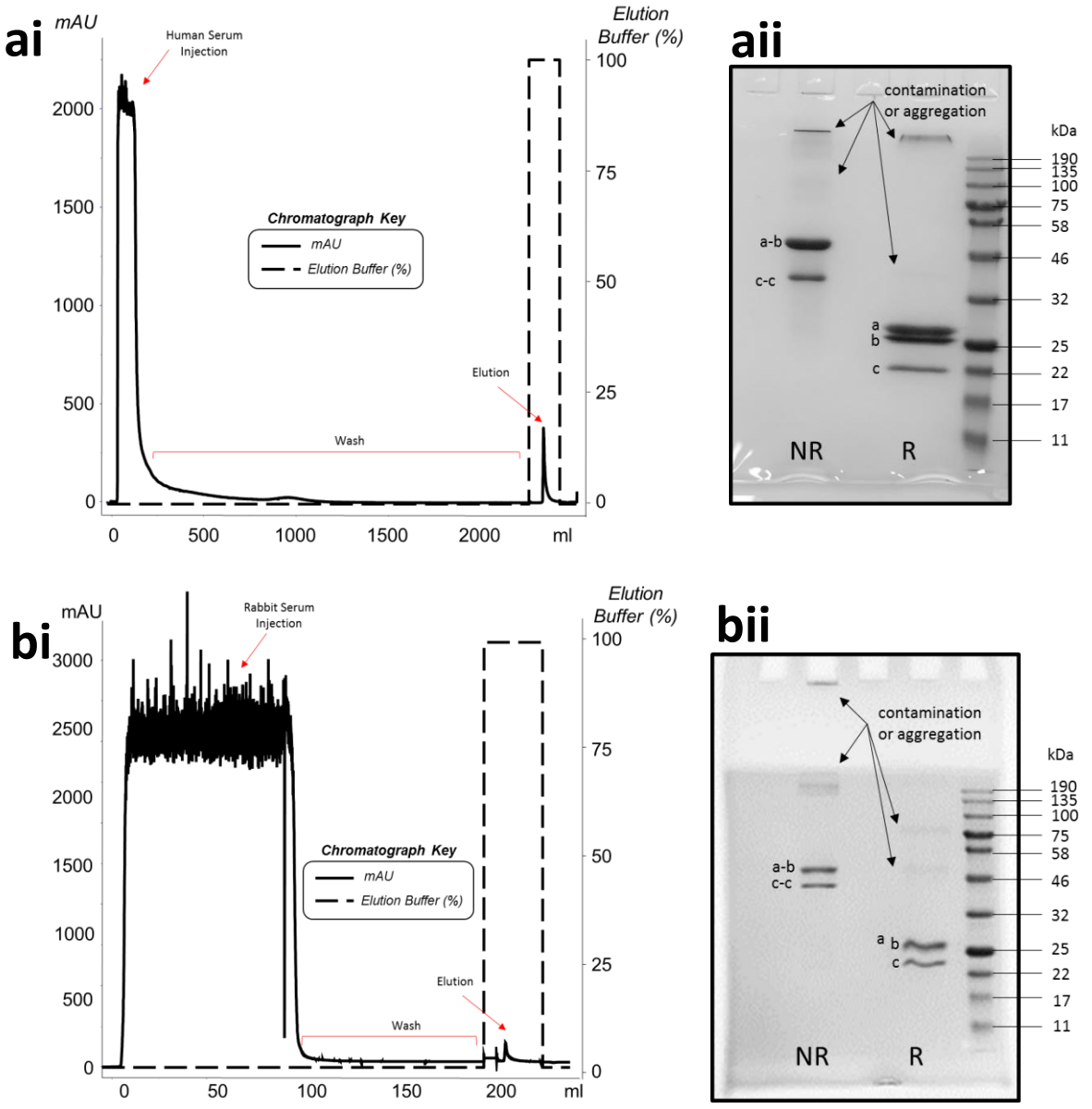


Figure 4. 10 Isolation of Human and Rabbit C1q – Affinity Chromatography

Human C1q was purified by cation exchange. ***ai***, Typical chromatograph showing injection of human serum through BioRex 70 resin and elution of bound C1q. Rabbit C1q was purified from by affinity chromatography. ***bi***, Typical chromatograph showing injection of human serum through an immune complex column and elution of bound C1q. The purity and composition of human (***aii***) and rabbit (***bii***) C1q was assessed by SDS-PAGE. 500ng of human and rabbit C1q was run through a 12.5% polyacrylamide gel under non-reducing (NR) and reducing (R) conditions and stained with coomassie Brilliant Blue dye. Under non-reducing conditions, human and rabbit C1q appeared as two bands corresponding to the a-b and c-c dimers of C1q (labelled). Under reducing conditions, human and rabbit C1q appeared as three bands corresponding to the a, b and c monomers of C1q (labelled). In both preparations, other protein bands (assumed to aggregates of C1q and other contaminants) were also detected. These data showed that further purification of both human and rabbit C1q was necessary.

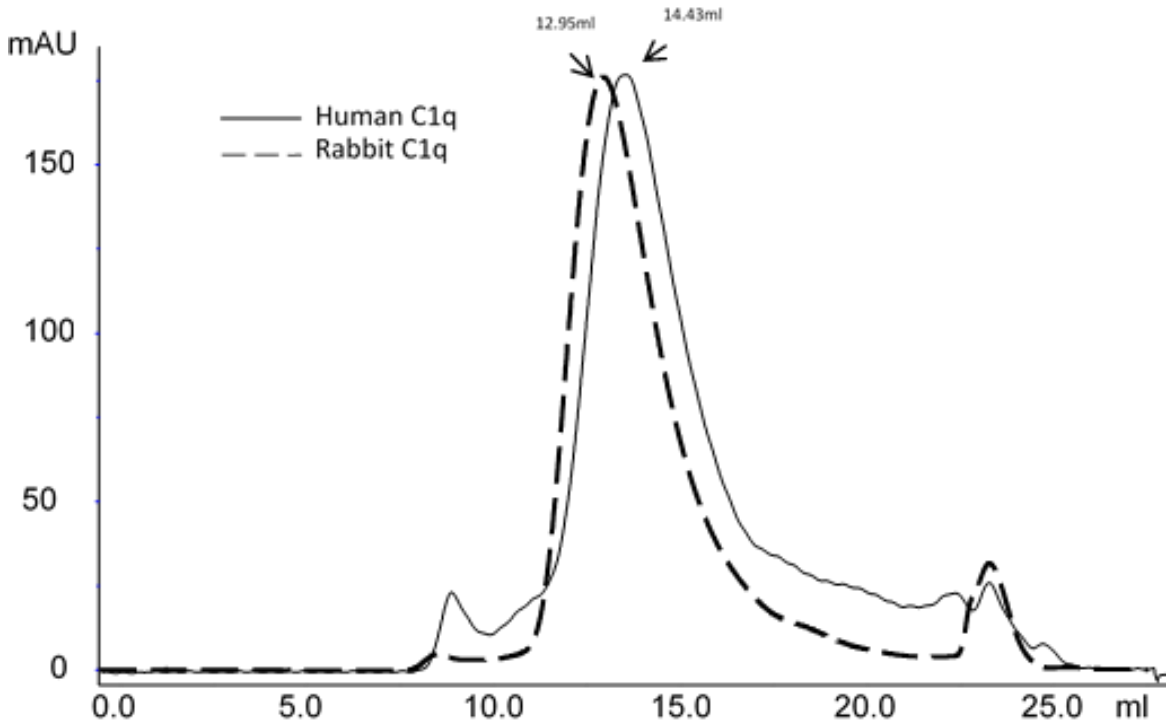


Figure 4. 11 Purification of Human and Rabbit C1q – Size Exclusion Chromatography
Human C1q was purified by cation exchange by injection of human serum through BioRex 70 resin. Rabbit C1q was purified from by affinity chromatography by injection of rabbit serum through a Sepharose column conjugated with crosslinked Ig (rabbit anti-human IgG bound to human IgG). By SDS-PAGE analysis, it was noted that both human and rabbit C1q preparations contained both aggregates of C1q and other contaminants (detailed in ***Figure 4.10***). To remove these contaminants human and rabbit C1q preparations were injected over a 24ml Superdex 75 size exclusion chromatography column. Approximately 4-5mg (in 0.5ml buffer) rabbit or human C1q was injected over a 24ml Superdex 75 (SD75) column. An overlay of typical chromatographs displaying absorbance (mAU) over eluted volume (mL) following injection of human (solid line) and rabbit (broken line) C1q preparations are shown. The peak of human C1q elution occurred at 14.43ml (or 0.60 column volumes) whereas the peak of rabbit C1q occurred at 13.0ml (0.54 column volumes). This difference in elution peaks between human and rabbit C1q conclude that rabbit C1q is of a larger molecular weight that human C1q.

Once contaminating proteins were removed, both human and rabbit C1q preparations were run through a 12.5% polyacrylamide gel and stained with coomassie Brilliant Blue to assess purity and composition (***Figure 4. 12***). Under non-reducing conditions human and rabbit C1q appeared as two distinct bands, representing the a-b and c-c dimer subunits from which C1q is composed. Using the molecular weight marker (*ladder*) as a standard, the apparent molecular weights of the a-b heterodimer subunits of human and rabbit C1q were interpolated as 58.9kDa and 59.9kDa, respectively. The apparent molecular weights of the c-c homodimer subunits of human and rabbit C1q were interpolated as 42.6kDa and 52.0kDa, respectively. Under reducing conditions, human and rabbit C1q appeared a three bands, representing the a, b and c monomer subunits of C1q. The apparent molecular weights of the a, b and c chains of human C1q were calculated as 29.3kDa, 26.5kDa and 20.1kDa, respectively. The apparent molecular weights of the a, b and c chains of rabbit C1q were calculated as 27.3kDa, 25.9kDa and 22.0kDa, respectively. With knowledge of the structure of C1q (six a, b and c monomers), the molecular weights of human C1q was calculated as 468.5kDa and the molecular weight of rabbit C1q was calculated as 483.2kDa. No contaminating proteins were detected in either preparations meaning the concentration of human and rabbit C1q could be accurately calculated for the SPR assays.

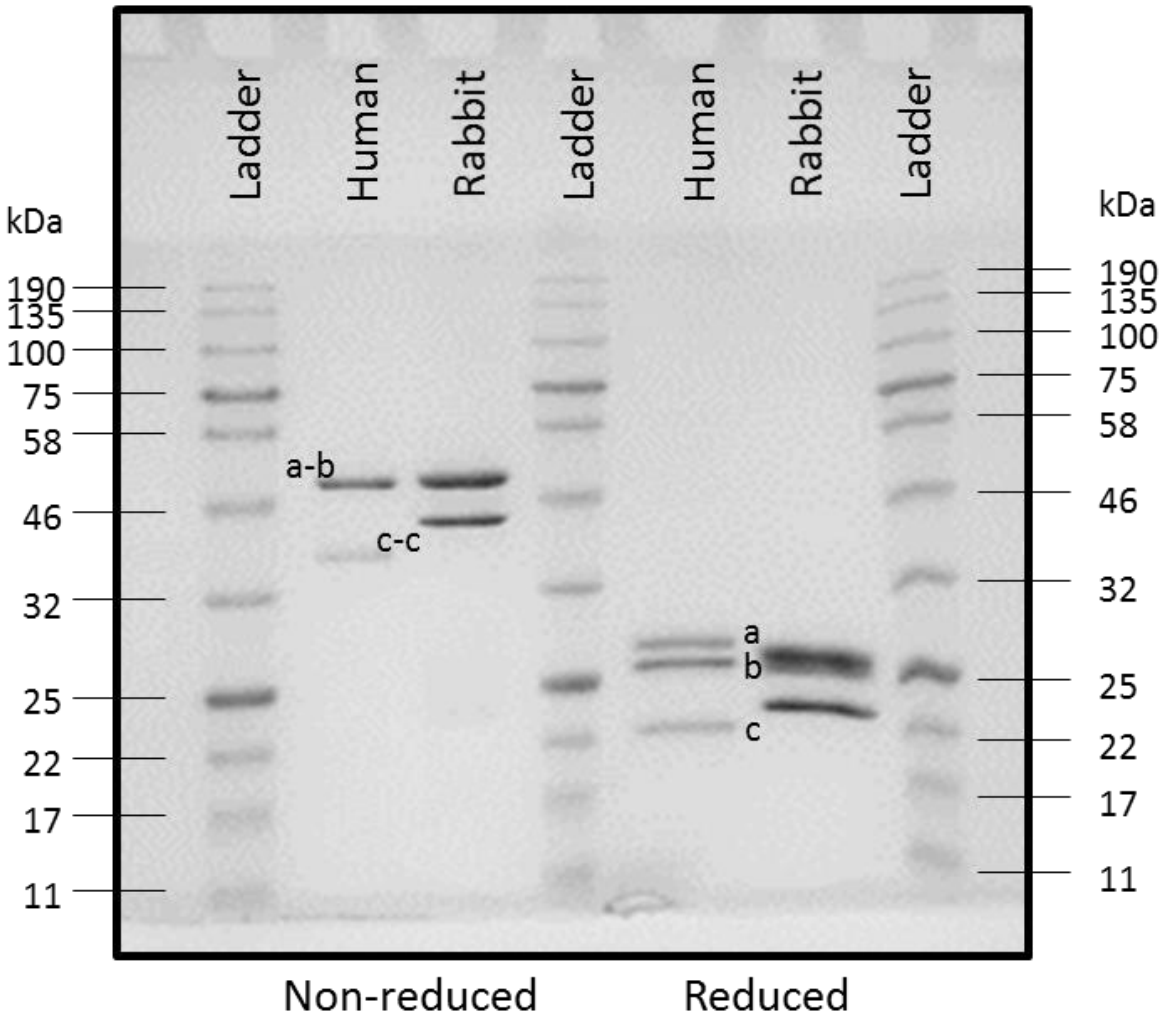


Figure 4. 12 Coomassie Stain of Human and Rabbit C1q

Human C1q was purified by cation exchange by injection of human serum through BioRex 70 resin (detailed in ***Figure 4.10***). Rabbit C1q was purified from by affinity chromatography by injection of rabbit serum through a Sepharose column conjugated with crosslinked Ig (rabbit anti-human IgG bound to human IgG). Contaminating proteins, aggregates and fragments from both rabbit and human C1q preparations were then removed by injection over a 24ml Superdex 75 size exclusion chromatography column (detailed in ***Figure 4.11***). The purity and composition of each C1q preparation was assessed by SDS-PAGE. 500ng of purified C1q was run through a 12.5% polyacrylamide gel under non-reducing and reducing conditions and stained with coomassie Brilliant Blue dye. Molecular weight markers (MwM) were run either side of the gel and used to interpolate the molecular weight (kDa) of each protein band. Under non-reducing conditions, human and rabbit C1q appear as two bands corresponding to the a-b (human = 58.9kDa, rabbit= 59.9kDa) and c-c (human = 42.6kDa, rabbit= 52.0kDa) dimers from which C1q is composed. Human and rabbit C1q reduced into three band corresponding to the a (human = 29.3kDa, rabbit= 27.3kDa), b (human = 26.5kDa, rabbit= 25.9kDa) and c (human = 20.1kDa, rabbit= 22.0kDa) monomers from which C1q is composed. No contaminating proteins could be detected in any antibody preparation.

4.3.4 Assessment of Human and Rabbit C1q Affinity to Antibody Subclasses by SPR

The affinity of human and rabbit C1q to human IgG1, IgG2, IgG3 and IgG4 antibody was measured by SPR as described by Patel et al., (2015). Recombinant Protein L was used to capture antibodies to the surface of a CM5 SPR sensor chip via the light chain (Björck, 1988). Protein L was amine-coupled to flow cells one and two at 2654RU and 2553.5RU, respectively. Flow cell 2 was used to capture antibody whilst flow cell 1 was used as the reference surface.

Human and rabbit C1q was then injected over captured IgG1, IgG2, IgG3, IgG4 and IgM antibody at a range of concentrations and binding (RU) measured (***Figure 4. 14***, ***Figure 4. 15***, ***Figure 4. 16*** and ***Figure 4. 17***). The amount of each antibody captured was adjusted in order to equalise maximum human and rabbit C1q binding (***Figure 4. 13***). Unfortunately, protein L-captured human IgM antibody did not interact with C1q (***Figure 4. 13e***). As such, human IgM was excluded from further SPR affinity analyses. As will be discussed below, it is expected that the conformation of human IgM antibody remains planar when bound by protein L and thus the C1q binding sites of IgM remain inaccessible.

The KD of human and rabbit C1q to each antibody preparation was then calculated by steady-state analysis (***Table 4.1***). The KD of human C1q with human IgG1 was calculated as 136.8nM, IgG2 as 198.1nM, IgG3 as 37.69nM and IgG4 as 358.1nM. The KD of rabbit C1q with human IgG1 was calculated as 196.2nM, IgG2 as 231.8nM, IgG3 as 123.1nM, IgG4 as 378.0nM and rabbit IgG as 219.3nM.

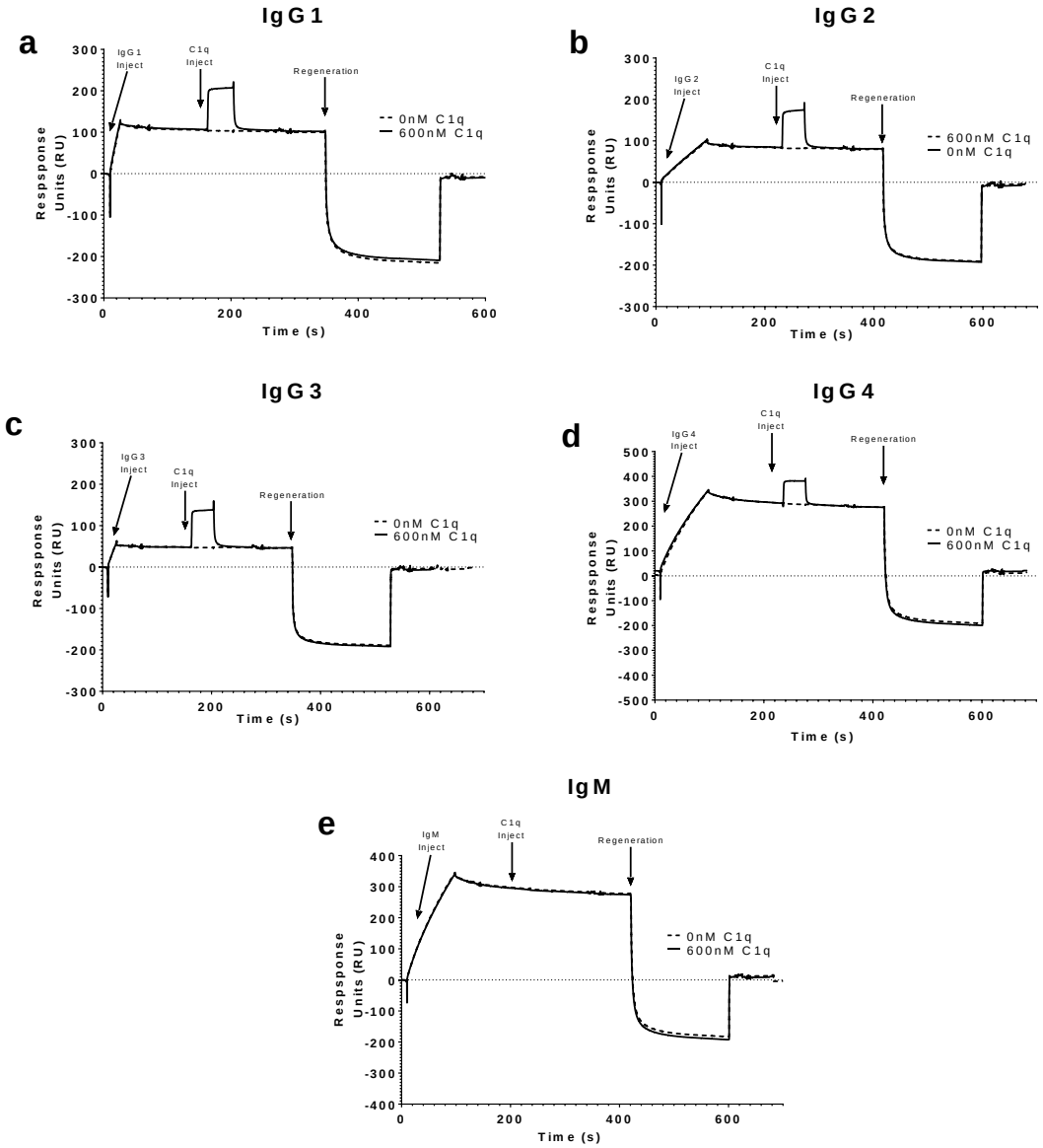


Figure 4. 13 Human C1q Binding to each antibody isotype

To investigate the differences in the interaction of human and rabbit C1q to the human IgG subclasses, the affinity of human and rabbit C1q to human IgG1, IgG2, IgG3, IgG4 and IgM antibodies was measured by surface plasmon resonance (SPR). Human IgG1, IgG2, IgG3, IgG4 and IgM antibodies were purified from human plasma by affinity chromatography (as detailed in ***Figure 4.6***). Human and rabbit C1q were purified from serum by affinity chromatography and cation exchange chromatography, respectively (as detailed in ***Figure 4.12***). Firstly, the ability of human C1q to bind protein L-captured human IgG1, IgG2, IgG3, IgG4 and IgM antibodies was assessed. The purified human IgG1, IgG2, IgG3 or IgG4 antibodies were captured to a protein L-coated CM5 Biacore™ sensor chip. Once captured, either buffer (0nM C1q) human C1q (600nM C1q) was injected over each antibody and binding was assessed. Sensograms showing the binding achieved (response units (RU)) with either 0nM C1q (broken line) or 600nM C1q (solid line) over time (seconds (s)) with protein L-captured human IgG1 (***a***), IgG2 (***b***), IgG3 (***c***), IgG4 (***d***) and IgM (***e***) are shown. Antibodies were injected at 0s and human C1q was injected between 160s and 220s for 40 seconds. Chip surfaces were regenerated between 360s and 420s for 180 seconds. C1q injection of over protein L-captured antibody subclasses produced 99RU (IgG1; ***a***), 89RU (IgG2; ***b***), 88RU (IgG3; ***c***) and 89RU (IgG4; ***d***). Human C1q did not bind protein L-captured human IgM (***e***).

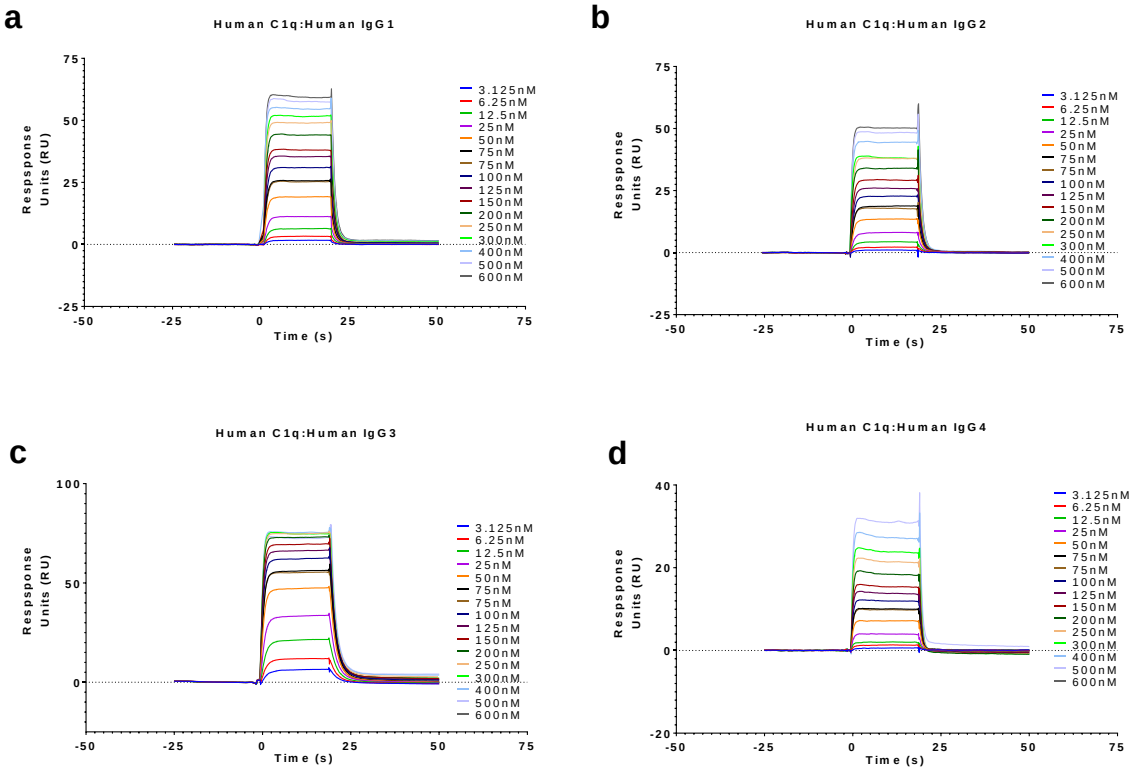


Figure 4. 14 Sensorgrams of Human C1q Binding to Immobilised Antibody Subclasses
To investigate the differences in the interaction of human and rabbit C1q to the human IgG subclasses, the affinity of human and rabbit C1q to human IgG1, IgG2, IgG3 and IgG4 antibodies was measured by surface plasmon resonance (SPR). Human IgG1, IgG2, IgG3 and IgG4 antibodies were purified from human plasma by affinity chromatography (as detailed in ***Figure 4.6***). Human and rabbit C1q were purified from serum by affinity chromatography and cation exchange chromatography, respectively (as detailed in ***Figure 4.12***). The purified human IgG1, IgG2, IgG3 or IgG4 antibodies were captured to a protein L-coated CM5 Biacore™ sensor chip. Once captured, human or rabbit C1q was injected over each antibody at a range of different concentrations (600-3.125nM). Human and rabbit C1q binding to each antibody subclass was assessed at each. Sensograms showing the binding achieved (response units (RU)) at each concentration of purified human C1q over time (seconds (s)) with protein L-captured human IgG1 (***a***), IgG2 (***b***), IgG3 (***c***) and IgG4 (***d***) are shown. Human C1q was injected at time zero for 20 seconds. Each line represents one injection of C1q at the concentration listed in the key for each sensogram.

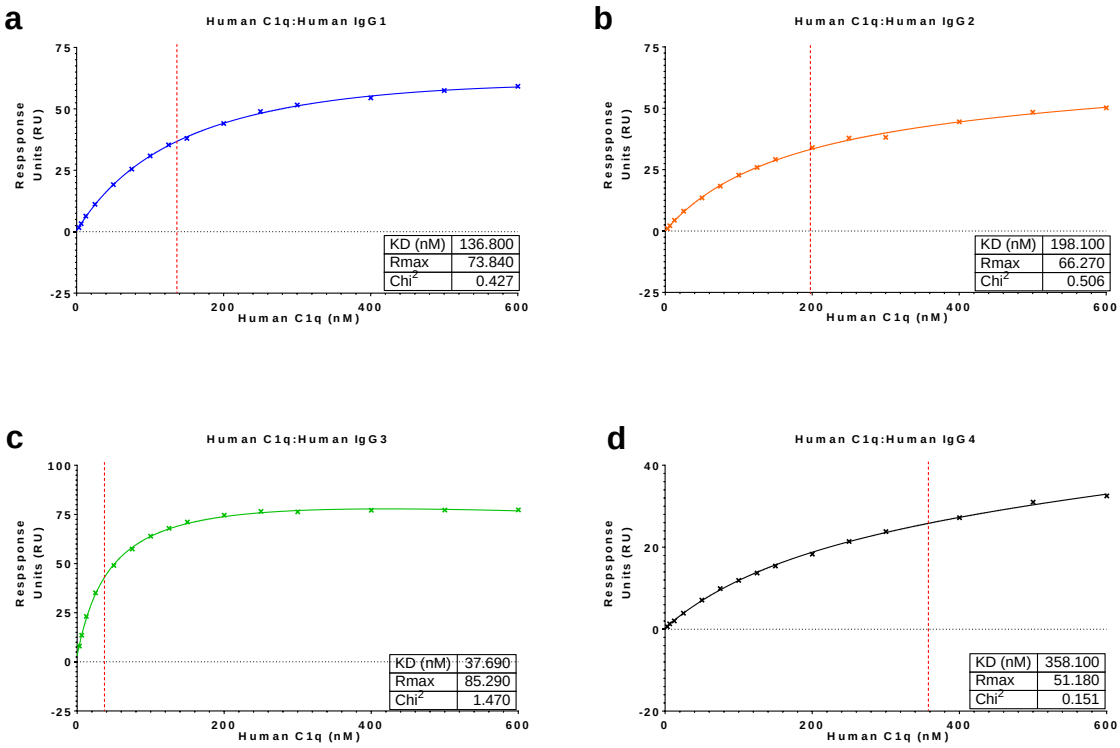


Figure 4. 15 Steady state analysis of Human C1q interaction with different Antibody Subclasses

To investigate the differences in the interaction of human and rabbit C1q to the human IgG subclasses, the affinity of human and rabbit C1q to human IgG1, IgG2, IgG3 and IgG4 antibodies was measured by surface plasmon resonance (SPR). Human IgG1, IgG2, IgG3 and IgG4 antibodies were purified from human plasma by affinity chromatography (as detailed in ***Figure 4.6***). Human and rabbit C1q were purified from serum by affinity chromatography and cation exchange chromatography, respectively (as detailed in ***Figure 4.12***). The purified human IgG1, IgG2, IgG3 or IgG4 antibodies were captured to a protein L-coated CM5 Biacore™ sensor chip. Once captured, human or rabbit C1q was injected over each antibody at a range of different concentrations (600-3.125nM). The dissociation constants (KD) of human and rabbit C1q for each antibody subclass were interpolated by steady state analysis (i.e. the concentration (nM) of C1q achieving 50% of maximum C1q binding for an antibody subclass). Graphs showing the binding achieved (response units (RU)) at each concentration of purified human C1q with protein L-captured human IgG1 (***a***), IgG2 (***b***), IgG3 (***c***) and IgG4 (***d***) are shown. Each point represents RU at each concentration of purified human C1q. The solid line represents the steady state curve fitted to each data set. The red, broken line represents the concentration of C1q achieving 50% of maximum C1q binding for an antibody subclass. ***Inserts***, The KD (nM), chi-squared (goodness of fit; Chi^2) and maximum C1q binding (Rmax) values for human C1q to each antibody preparation are detailed.

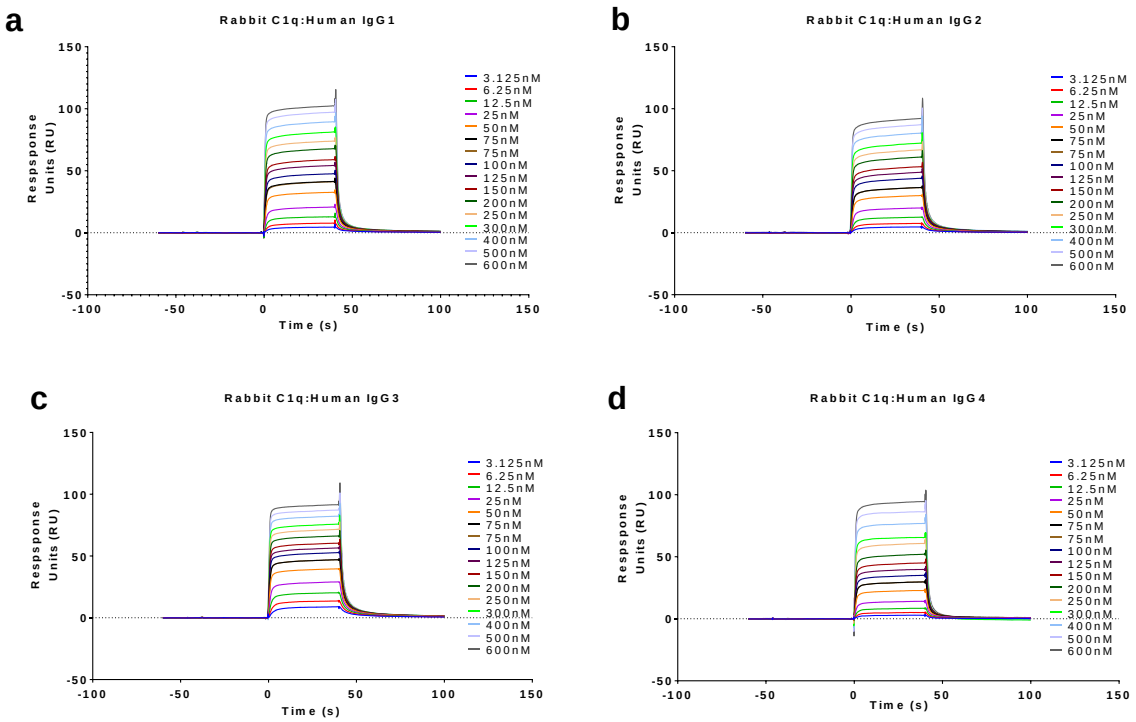


Figure 4. 16 Sensorgrams of Rabbit C1q Binding to Immobilised Antibody Subclasses
To investigate the differences in the interaction of human and rabbit C1q to the human IgG subclasses, the affinity of human and rabbit C1q to human IgG1, IgG2, IgG3 and IgG4 antibodies was measured by surface plasmon resonance (SPR). Human IgG1, IgG2, IgG3 and IgG4 antibodies were purified from human plasma by affinity chromatography (as detailed in ***Figure 4.6***). Human and rabbit C1q were purified from serum by affinity chromatography and cation exchange chromatography, respectively (as detailed in ***Figure 4.12***). The purified human IgG1, IgG2, IgG3 or IgG4 antibodies were captured to a protein L-coated CM5 Biacore™ sensor chip. Once captured, human or rabbit C1q was injected over each antibody at a range of different concentrations (600-3.125nM). Human and rabbit C1q binding to each antibody subclass was assessed at each. Sensograms showing the binding achieved (response units (RU)) at each concentration of purified rabbit C1q over time (seconds (s)) with protein L-captured human IgG1 (***a***), IgG2 (***b***), IgG3 (***c***) and IgG4 (***d***) are shown. Rabbit C1q was injected at time zero for 40 seconds. Each line represents one injection of C1q at the concentration listed in the key for each sensogram.

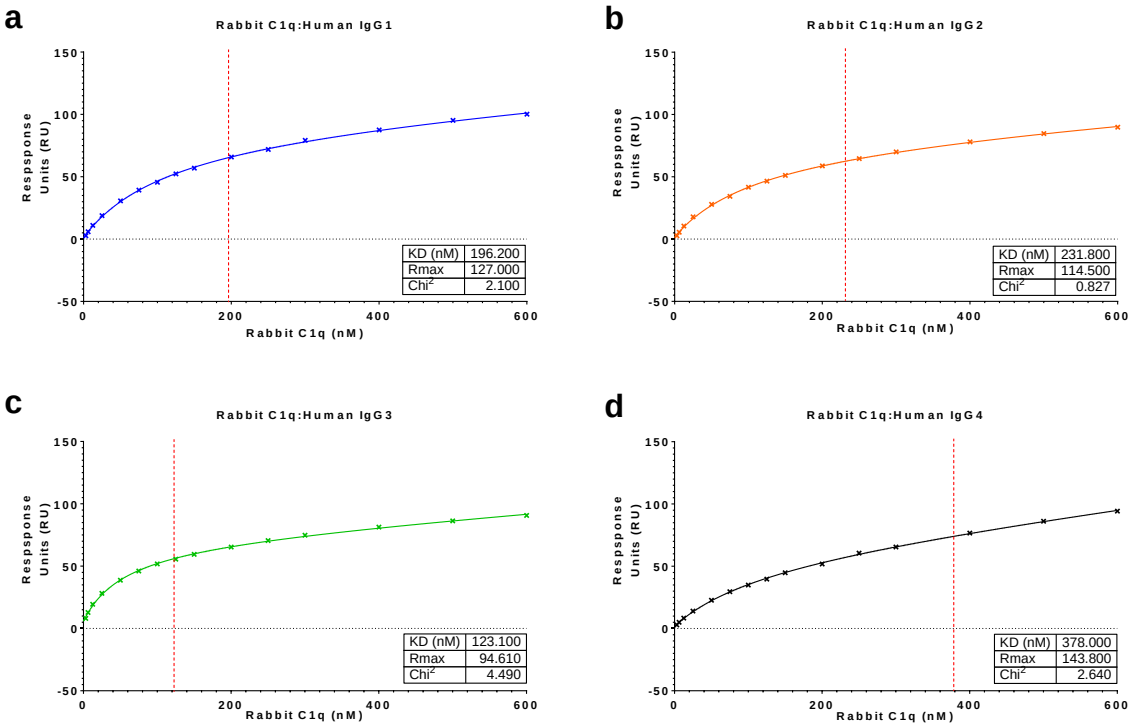


Figure 4. 17 Steady state analysis of Human C1q interaction with different Antibody Subclasses

A To investigate the differences in the interaction of human and rabbit C1q to the human IgG subclasses, the affinity of human and rabbit C1q to human IgG1, IgG2, IgG3 and IgG4 antibodies was measured by surface plasmon resonance (SPR). Human IgG1, IgG2, IgG3 and IgG4 antibodies were purified from human plasma by affinity chromatography (as detailed in ***Figure 4.6***). Human and rabbit C1q were purified from serum by affinity chromatography and cation exchange chromatography, respectively (as detailed in ***Figure 4.12***). The purified human IgG1, IgG2, IgG3 or IgG4 antibodies were captured to a protein L-coated CM5 Biacore™ sensor chip. Once captured, human or rabbit C1q was injected over each antibody at a range of different concentrations (600-3.125nM). The dissociation constants (KD) of human and rabbit C1q for each antibody subclass were interpolated by steady state analysis (i.e. the concentration (nM) of C1q achieving 50% of maximum C1q binding for an antibody subclass). Graphs showing the binding achieved (response units (RU)) at each concentration of purified rabbit C1q with protein L-captured human IgG1 (***a***), IgG2 (***b***), IgG3 (***c***) and IgG4 (***d***) are shown. Each point represents RU at each concentration of purified rabbit C1q. The solid line represents the steady state curve fitted to each data set. The red, broken line represents the concentration of C1q achieving 50% of maximum C1q binding for an antibody subclass. ***Inserts***, The KD (nM), chi-squared (goodness of fit; Chi^2) and maximum C1q binding (Rmax) values for rabbit C1q to each antibody preparation are detailed.

Table 4. 1 Dissociation Constants (KD) of Rabbit and Human C1q for each Antibody Subclass

To investigate the differences in the interaction of human and rabbit C1q to the human IgG subclasses, the affinity of human and rabbit C1q to human IgG1, IgG2, IgG3 and IgG4 antibodies was measured by surface plasmon resonance (SPR). Human IgG1, IgG2, IgG3 and IgG4 antibodies were purified from human plasma by affinity chromatography (as detailed in ***Figure 4.6***). Human and rabbit C1q were purified from serum by affinity chromatography and cation exchange chromatography, respectively (as detailed in ***Figure 4.12***). The purified human IgG1, IgG2, IgG3 or IgG4 antibodies were captured to a protein L-coated CM5 Biacore™ sensor chip. Once captured, human or rabbit C1q was injected over each antibody at a range of different concentrations (600-3.125nM). Human and rabbit C1q binding to each antibody subclass was assessed at each concentration (sensograms for these assays are detailed in ***Figure 4.14*** and ***Figure 4.16***). The dissociation constants (KD) of human and rabbit C1q for each antibody subclass was calculated by steady state analysis (i.e. the concentration (nM) of C1q achieving 50% of maximum C1q binding for an antibody subclass) as detailed in ***Figure 4.15*** and ***Figure 4.17***. The KD (nM) and Chi-squared (goodness of fit) values for human and rabbit C1q to each antibody preparation is shown in the table below.

Antibody	Human C1q		Rabbit C1q	
	KD; nM	*Chi Squared*	*KD; nM*	*Chi Squared*
IgG1	136.8	2.1	196.2	0.35
IgG2	198.1	0.83	231.8	0.11
IgG3	37.69	2.59	123.1	0.82
IgG4	358.1	2.64	378	0.15

4.4 Discussion

4.4.1 Interaction of Antibody Subclasses with Meningococcal Polysaccharides

I have investigated the functional affinity of IgG1, IgG2, IgG3, IgG4 and IgM antibodies in plasma to meningococcal polysaccharides (serogroups A, C, W and Y) by competitive ELISA (***Figure 4. 2***). The functional affinity of IgG1 and IgM antibody was significantly higher than IgG2 and IgG4 antibody. The functional affinity of IgG3 antibody was significantly higher than IgG4 antibody.

The functional affinities of IgG1 antibody either in plasma or purified from plasma were directly compared to rule out any artefacts caused by antibodies of other subclasses, other than the one being assessed (***Figure 4. 4***). There was no significant difference between the functional affinities of plasma IgG1 and purified IgG1 antibodies and it was concluded that the presence of other antibody subclasses did not alter functional affinity.

The functional affinity of two anti-FH antibodies, whose affinity had been previously assessed by SPR, was measured by competitive ELISA (***Figure 4. 5***). Functional affinity and KD values correlated well further validating the competitive ELISA as a method to compare the relative functional affinity of antibodies to antigen. It may be argued that comparing the affinity of a monoclonal antibody to a protein antigen by competitive ELISA and SPR may not be directly relevant as a way of validating the use of competitive ELISA to measure the affinity of polyclonal antibody to a polysaccharide antigen. In this way, it may be useful to further validate the competitive ELISA for this specific purpose by comparing the affinity of purified meningococcal polysaccharides (serogroups A, C, W and Y) specific antibody to meningococcal polysaccharides by SPR to the values achieved by the competitive ELISA.

The affinity of antibodies induced by meningococcal polysaccharide vaccines is thought to be important in protection against disease, with antibodies of a higher affinity producing higher SBA titres (Hetherington and Lepow, 1992; Schlesinger et al., 1992). A previous study has shown that the concentration of meningococcal polysaccharide-specific IgG1 antibody in vaccinee plasma positively correlated with overall antibody affinity to meningococcal polysaccharides and that the concentration IgG2 antibody negatively correlated with antibody affinity (de Voer et al., 2011). This study has shown that meningococcal polysaccharide-specific IgG1 antibody, as well as IgM antibody, was of a higher affinity than meningococcal polysaccharide-specific IgG2 antibody. These data reveal the mechanism behind the positive correlation between concentration of IgG1 antibody and affinity of antibody to meningococcal polysaccharides and the mechanism behind the negative correlation between concentration of IgG2 antibody and affinity of antibody to meningococcal polysaccharides.

4.4.2 Interaction of Human and Rabbit C1q with different Antibody Subclasses

4.4.2.1 Human and Rabbit C1q Binding ELISA

The interaction of human and rabbit C1q to various antibody preparations was assessed by ELISA (***Figure 4. 9***). By rank order, human C1q bound most to IgG3 antibody followed by IgG1 >> IgM > IgG2 > IgG4. The differential ability of IgM and human IgG subclasses to bind C1q are well known and mirror the data produced here (Bindon et al., 1988; Brüggemann et al., 1987; Kaul and Loos, 1997; Schumaker et al., 1976). In line with the rank order of human C1q binding to antibodies, rabbit C1q bound most to IgG3 antibody followed by IgG1 > IgM >> IgG2 > IgG4.

Human and rabbit C1q binding to human IgG, rabbit IgG and human anti-MenACWY antibody was also assessed in the same ELISA (***Figure 4. 9***). With these antibody preparations included, the rank order of human C1q binding was: IgG3 > human IgG > IgG1 > rabbit IgG > anti-MenACWY > IgM > IgG2 > IgG4. The rank order of rabbit C1q binding to antibody preparations was: IgG3 > human IgG > IgG1 > IgM > rabbit IgG > anti-MenACWY > IgG2 > IgG4. Rabbit IgG consists of only one subclass whereas the human IgG and anti-MenACWY antibody are composed of multiple antibody subclasses (***Figure 4. 8***). Although the hinge length of rabbit IgG is one residue smaller than IgG2 and IgG4 (eleven versus twelve), both isotypes which interact poorly with C1q, it shows intermediate segmental flexibility between that of IgG1 and IgG4 (Dangl et al., 1988). The extended 'upper' hinge region of rabbit IgG accounts for this seemingly disproportionate flexibility and enhanced ability to interact with C1q compared to that of IgG2 and IgG4 (Dangl et al., 1988; Rayner et al., 2012). With all this taken into account, the amount of C1q bound to purified human IgG, rabbit IgG and anti-MenACWY antibody preparations in this assay is as expected.

Previous studies show that the bactericidal activity of human IgM towards *Neisseria meningitidis* in the presence of rabbit serum is up to 1000 times greater than with human serum. For comparison, the bactericidal activity of human IgG towards *Neisseria meningitidis* in the presence of rabbit serum is only 10 times greater than with human serum (Griffiss and Goroff, 1983; Mandrell et al., 1995; Santos et al., 2001; Zollinger and Mandrell, 1983). In this study, significant rabbit C1q binding was measured with human IgG3, IgG1 and IgM antibodies whereas significant human C1q binding was measured with human IgG3 and IgG1 antibodies only (***Figure 4. 9***). In this study I have confirmed that human IgM antibody binds rabbit C1q better than human C1q, and that this would result in better activation of rabbit complement than human complement, and correlates with the functional assays previously described.

4.4.2.2 Assessment of Human and Rabbit C1q Affinity to Antibody Subclasses by SPR

The affinity of human and rabbit C1q to human IgG1, IgG2, IgG3 and IgG4 antibody was measured by SPR using a method previously described by Patel et al., (2015) (***Table 4.1***). Unlike other commonly used immunoglobulin binding proteins that bind to the Fc region, such as Protein A and Protein G, Protein L binds to the light chain of antibody (Graille et al., 2001). The binding sites of Protein G and Protein A for antibody spans between the CH2 and CH3 domains of the Fc region (Deisenhofer, 1981; Kato et al., 1995). This binding site overlaps with the binding site of C1q and therefore capable of disrupting C1q binding to antibody making their use in studying the affinity of immunoglobulins to C1q inappropriate (Nitsche-Schmitz et al., 2007). In contrast, Protein L binds to the light chain of immunoglobulins minimising interference with C1q binding (Björck, 1988; Patel et al., 2015).

Human C1q was purified from human serum by cation exchange chromatography using a method first described by Tenner et al., (Tenner et al., 1981). Rabbit C1q was isolated from rabbit serum by affinity chromatography based on a method described by Pohl et al., (Pohl et al., 1980). Rabbit C1q could not be isolated by cation exchange chromatography. The exact mechanism behind this is unknown but differences in the charge of the two molecules is probable (Lowe and Reid, 1974; Reid et al., 1972). By SDS-PAGE analysis, it was shown that rabbit C1q is of a larger molecular weight that human C1q (483.2kDa versus 468.5kDa) (***Figure 4. 12***). The a-b heterodimer subunit of human and rabbit C1q were of a similar molecular weight (58.9kDa versus 59.9kDa, respectively) whereas the c-c homodimer subunit of rabbit C1q was of a greater molecular weight than the c-c homodimer subunit (52.0kDa versus 42.6kDa, respectively). When injected over 24ml Superdex 75 size exclusion chromatography column rabbit C1q eluted earlier than human C1q, further emphasising the larger molecular weight of rabbit C1q (***Figure 4. 11***). These differences between rabbit and C1q have been previously

reported (Lowe and Reid, 1974; Reid et al., 1972; Swanson et al., 1988; Volanakis and Stroud, 1972).

Protein L-bound human IgM antibody did not interact with purified human C1q (***Figure 4. 13***). As such, the affinity of human and rabbit C1q to human IgM antibody could not be assessed. In solution, IgM exists in a planar conformation obscuring the C1q binding sites within the structure of the molecule. When bound to antigen, IgM flexes significantly about its CH2 region. This altered, 'staple' conformation reveals the C1q binding site on the CH3 and CH4 domains of IgM allowing C1q to associate (Czajkowsky and Shao, 2009; Feinstein and Munn, 1969; Perkins et al., 1991; Zlatarova et al., 2006). The conformation of IgM bound to Protein L has not been studied but based on these data it is predicted to remain planar keeping the C1q binding sites inaccessible. The affinity of human and rabbit C1q to human IgM would be of great interest for this project as the greatest difference in complement activation between human and rabbit complement is seen with IgM (Mandrell et al., 1995; Santos et al., 2001).

Two methods were attempted to reveal the C1q binding sites on IgM with limited success (data not shown). The first method attempted to isolate the IgM monomer fraction by partially reducing purified human IgM (pentameric) with 10mM 2-mercaptoethanol as described by Ditzel et al., (1993). Following incubation of IgM with 2-mercaptoethanol, approximately 17% IgM reduced to monomeric IgM with the remaining IgM reducing to trimeric IgM (27%), dimeric IgM (13%), half-monomeric IgM (36%) and other unidentified products (7%). The IgM monomer fraction was separated from the other products of reduction by size exclusion chromatography by injection through a superose 6 column. The final product (composed of 67% IgM monomer and 33% IgM half-monomer) was captured onto the protein L-coated CM5 chip and C1q binding was assessed by SPR. Again, no binding of C1q to the protein L-captured IgM monomer and half-monomer preparation could be detected. It was concluded that this method of isolating monomeric IgM was inappropriate as either: the IgM monomer remains planar when bound to

protein L, keeping the C1q binding sites inaccessible, or the C1q binding sites of the IgM monomer were denatured when incubated with 10mM 2-mercaptoethanol. More appropriate methods of isolating monomeric IgM may prove more successful. The second method attempted to induce a conformational change of IgM antibody from the 'planar' to the 'staple' conformation by capturing purified anti-TT IgM onto a CM5 chip by coating the chip with TT. In this way, the C1q binding site on the CH3 and CH4 domains of IgM will be revealed allowing C1q to associate (Czajkowsky and Shao, 2009; Feinstein and Munn, 1969; Perkins et al., 1991; Zlatarova et al., 2006). The purified anti-TT IgM bound well to the TT-coated CM5 chip but no IgM specific C1q binding could be detected due to high levels of non-specific binding to the TT-coated reference CM5 chip. Using a more appropriate antigen to capture antigen-specific IgM may prove more successful.

The amount of each antibody captured to protein L-coated CM5 SPR sensor chip was adjusted in order to equalise maximum human and rabbit C1q binding (***Figure 4. 13***). A much greater RU of captured IgG4 antibody was needed to reach an Rmax equivalent to that of IgG3 antibody. A lower RU of IgG2 than IgG4 antibody and a lower RU of IgG1 than IgG2 antibody was required to reach an equal C1q Rmax achieved with IgG3 antibody. C1q is able to bind up to six molecules of IgG. The affinity of C1q to antibody and resulting complement activation increases with the number IgG molecules engaged (Diebolder et al., 2014; Wright et al., 1980). It has also been shown that antigen density also dictates the level of complement activation achieved by an antibody subclass (Garred et al., 1989; Giuntini et al., 2016). IgG3 antibody activates complement better than IgG1 antibody when bound to a sparsely expressed antigen whereas IgG1 antibody activates complement better than IgG3 antibody when bound to a densely expressed antigen (Giuntini et al., 2016). The differential densities of antibody subclasses (IgG4 > IgG2 > IgG1 > IgG3) may have altered their relative affinities to human and rabbit C1q. If this

is the case, the relative affinity of IgG4 antibody to C1q will have been increased compared to IgG2 < IgG1 < IgG3 antibodies.

By rank order, human C1q had the highest affinity to human IgG3 antibody followed by IgG1, IgG2 and IgG4 (***Table 4.1***). These results are in line with previous studies on the difference in affinity of human IgG subclasses to human C1q (Bindon et al., 1988; Brüggemann et al., 1987; Kaul and Loos, 1997; Patel et al., 2015; Schumaker et al., 1976). By ultracentrifugation analysis, Schumaker et al., (1976) reported the affinity of human C1q to monomeric antibody subclasses as 85µM (IgG1), 157µM (IgG2), 34µM (IgG3) and 229µM (IgG4). In the study by Patel et al., (2015), from which this assay is based upon, the KD of human C1q to human IgG1, IgG2, IgG3 and IgG4 was calculated as 81nM, 191nM, 88nM and 223nM respectively.

The rank order of rabbit C1q affinity to human IgG subclasses were the same as seen with human C1q (***Table 4.1***). In general, the KD of rabbit C1q to human IgG subclasses were higher than with human C1q indicating an overall lower affinity. The greatest difference in KD between human and rabbit C1q was seen with IgG3 (ratio of 1:3.3). The next greatest difference in KD was IgG1 (1:1.4) followed by IgG2 (1:1.2) and IgG4 (1:1.1) with an average ratio of 1:1.8. No in-depth study comparing the differences between rabbit C1q with human C1q and their effects on the interaction with immunoglobulins been carried out previously. It is assumed that the differences seen here are as a result of differences in charge, size and binding motifs seen between human and rabbit C1q.

A study assessing the human and rabbit complement-fixing activities of human IgG subclasses showed that human IgG1 and IgG3 activated human complement significantly more than rabbit complement whereas and human IgG2 activated rabbit complement significantly more than human complement (Dangl et al., 1988). These data suggest that human C1q has a higher affinity to human IgG1 and IgG3 antibody and a lower affinity to human IgG2 antibody than rabbit C1q. The data from this study confirm that human C1q does show greater affinity for

human IgG1 and IgG3 antibody than rabbit C1q, but disagree with previous data by showing that human C1q has a greater affinity for human IgG2 than rabbit C1q. The difference between the affinity of human and rabbit C1q to human IgG2 antibody was small and the ability of human IgG2 to activate rabbit complement better than human complement may be as a result of a lower activation threshold for rabbit C1q.

4.4.2.3 Conclusions

In the previous chapter, I showed that rSBA titres significantly correlated with the concentration of serogroup-specific IgM antibody (***Chapter 3: Table 3.4b*** and ***Table 3.6***). In stark contrast, hSBA titres did not correlate well with the concentration of serogroup-specific IgM antibody. As such, I hypothesised that the differential affinity of human and rabbit C1q to human antibody subclasses and subsequent differential complement activation accounts for the poor correlation between hSBA and rSBA titres.

In this chapter, I have highlighted important differences between the interactions of human and rabbit C1q with human antibody subclasses, which may explain the mechanism behind the poor correlation between hSBA and rSBA titres. These differences were particularly apparent with the antibody subclasses IgM and IgG1 (and IgG3), which I have shown significantly correlate with rSBA and hSBA titres, respectively. Whether these differences in the interaction of human and rabbit C1q with human antibody subclasses results in differential human and rabbit complement activation will be investigated in the next chapter. Nevertheless, these data further question the use of rabbit serum as the source of complement in SBAs and provide significant insight into difficulties and challenges associated with interpretation of rSBA data.

Chapter Five – Contribution of Antibody Subclass and Complement Pathways to Bactericidal Killing with Rabbit or Human Complement

5.1 Introduction

5.1.1 Interaction of Antibody Subclasses with Human and Rabbit Complement

The classical pathway of complement activation is activated by IgG and IgM antibodies (Müller-Eberhard and Kunkel, 1961). The initiator of the classical pathway of complement is C1q, which is activated once bound to the Fc regions of IgM (staple) and multiple IgG antibodies (Gaboriaud et al., 2003; Idusogie et al., 2000; Moore et al., 2010; Morgan et al., 1995; Schneider and Zacharias, 2012; Thommesen et al., 2000; Xu et al., 1994). The human IgG subclasses differ in their ability to interact with C1q and activate complement (Ishizaka et al., 1967; Patel et al., 2015). Human IgG3 antibody expresses the highest affinity to C1q followed by IgG1, IgG2 and IgG4 (Patel et al., 2015; Schumaker et al., 1976). The staple form of IgM shows intermediate affinity for C1q in between that of IgG1 and IgG2 (Brüggemann et al., 1987). The difference in ability of human IgG subclasses to bind C1q is thought to be determined by combination of hinge length and flexibility, Fab steric hindrance, CH2 sequence, glycosylation in addition to antigen density and hexamer formation (Brekke et al., 1995; Diebolder et al., 2014; Tan et al., 1990; Xu et al., 1994).

The ability of human IgM and IgG subclasses to activate rabbit complement is poorly understood and it may be hypothesised that differences in the ability of human antibody subclasses to activate human and rabbit complement explains the poor correlation between hSBA and rSBA titres. Several studies have shown that human IgM antibody has a greatly enhanced bactericidal activity in the presence of rabbit serum compared to human serum than

other human antibody subclasses assessed (Mandrell et al., 1995; Santos et al., 2001; Zollinger and Mandrell, 1983). The concentration of human IgM antibody required for equivalent SBA titres is 500-1000 times less in the presence of rabbit serum compared to human serum. In contrast, the concentration of human IgG antibody required for equivalent SBA titres is only 10 times less in the presence of rabbit serum compared to human serum. In a study comparing the ability of human IgG subclasses to activate human and rabbit serum, the investigators showed that human IgG1 and IgG3 activates human complement better than rabbit complement whilst human IgG2 activates rabbit complement better than human (Dangl et al., 1988). Human IgG4 antibody did not activate human or rabbit complement.

In the first results chapter of this study, we showed that hSBA titres of immune serum correlated best with the concentration of *Neisseria meningitidis* specific IgG1 antibody concentrations. In contrast, rSBA titres of immune serum correlated best with the concentration of *Neisseria meningitidis* specific IgM antibody concentrations. These data suggested an important difference in the ability of human antibody subclasses to activate human and rabbit complement. In the second results chapter of this study, we showed that human IgG1 antibody binds human C1q better than rabbit C1q and that human IgM and IgG2 antibodies binds rabbit C1q better than human C1q. These data showed that there is a significant difference between the interaction of human subclasses with human and rabbit complement. Whether this difference in C1q binding between antibodies translates to a significant difference in the ability of antibody subclasses to activate human and rabbit serum will be investigated in this chapter.

5.1.2 Human and Rabbit Complement Pathways and Neisseria meningitidis

Complement-mediated lysis is considered by some to be the most important mechanism in the protection against invasive meningococcal disease (Goldschneider et al., 1969a; Granoff, 2009). Opsonisation by products of complement activation and subsequent phagocytosis of *Neisseria meningitis* in protection is less well defined but thought to play a significant role in some individuals (Granoff, 2009). High incidence rates of meningococcal disease in those deficient in terminal pathway components (C5, 6, 7, 8 and 9) and not in earlier components suggests greater importance of bacteriolysis over phagocytosis (Figueroa and Densen, 1991; Humphries et al., 2015; Maslanka et al., 1997).

Each pathway of complement activation has been implicated in the protection against meningococcal disease. Investigations on the relative importance of each complement pathway in the clearance of *Neisseria meningitidis* with human serum show that classical pathway activation, driven by antibody, is the most important pathway in the protection against meningococcal disease (Agarwal et al., 2014; Drogari-Apiranthitou et al., 2002). In individuals with lower concentrations of bactericidal antibody, an active alternative pathway, amplifying reduced classical pathway activation, plays an equally significant role in disease prevention (Hellerud et al., 2010; Ram et al., 2011). Although the lectin and alternative pathways are directly activated on the surface of *Neisseria meningitis*, in the absence of antibody their individual physiological relevance is questionable.

The importance of each complement pathway in the clearance of *Neisseria meningitidis* with rabbit serum is poorly understood and it may be hypothesised that species-specific differences in the interaction of the complement activation pathways with *Neisseria meningitidis* contribute to the poor correlation between hSBA and rSBA titres. *Neisseria meningitidis* is able to modulate the alternative pathway of complement activation by binding FH using a number of FH-specific proteins, including the FHbp. The specificity of FHbp to human

FH and not rabbit FH suggests that the alternative pathway of rabbit complement will be more active towards *Neisseria meningitidis* than the alternative pathway of human complement (Granoff et al., 2009). Further differences between human and rabbit complement activation on *Neisseria meningitidis* by each of the three pathways will be investigated in this chapter.

5.1.3 Chapter Aims

The aims of this chapter are as follows:

i. To compare the ability of human IgM and IgG subclasses to activate human and rabbit complement.
ii. To assess the contribution of the different complement cascade pathways to bactericidal killing with rabbit or human complement.

5.2 Activation of Human and Rabbit Complement by Different Subclasses of Human Antibody

It is hypothesised that the differential ability of human antibody subclasses to activate human and rabbit complement account for the poor correlation between SBA titres in assay using human or rabbit serum as the source of complement. In the previous chapter, I have shown that human and rabbit C1q (the initiator of the classical pathway of complement activation) differentially interact with human antibody subclasses. Whether these differences in interaction with human and rabbit C1q result in differences in the activation of human and rabbit complement will be investigated in this chapter.

5.2.1 Human and Rabbit Complement Deposition ELISA

Differences in the activation of human and rabbit complement by several purified antibody subclasses was measured by ELISA. Human and rabbit C3 deposition was detected as a marker of complement activation following the incubation of human or rabbit serum on ELISA plates coated with these purified antibody subclasses. Human and rabbit C3 deposition was detected using a sheep anti-human C3/C3c antibody.

In preparation for this ELISA, the cross-reactivity of a polyclonal sheep anti-human C3/C3c antibody (antibodies-online) to human and rabbit C3 was assessed by western blot (***Figure 5.1a***) Human and rabbit serum, diluted 1/400 (v/v) in PBS, was run through 7.5% polyacrylamide gel under non-reducing conditions, transferred onto a nitrocellulose membrane and probed with the polyclonal anti-C3-HRP conjugate antibody. Chemiluminescence was detected in both human and rabbit serum proving species cross-reactivity of the anti-C3 detection antibody and suitability for this and subsequent assays. The C3-positive band present in rabbit serum was noticeably larger than with human serum.

An active source of human and rabbit complement was required for this ELISA. The complement activity of human and rabbit serum was assessed by a classical pathway haemolytic assay ***Figure 5. 1b***. The human serum was prepared in house from a pool of three healthy adult donors. The frozen rabbit serum was purchased from VHBio (Gateshead, UK). A range of human and rabbit serum concentrations (10-0.04%), water only (positive) or buffer only (negative) was incubated with antibody sensitised sheep erythrocytes in duplicate. Subsequent haemolysis (405nm) at each concentration of rabbit and human serum was measured and percentage haemolysis calculated. It was concluded that both the human and rabbit serum were suitable sources of active complement for use in this and subsequent assays.

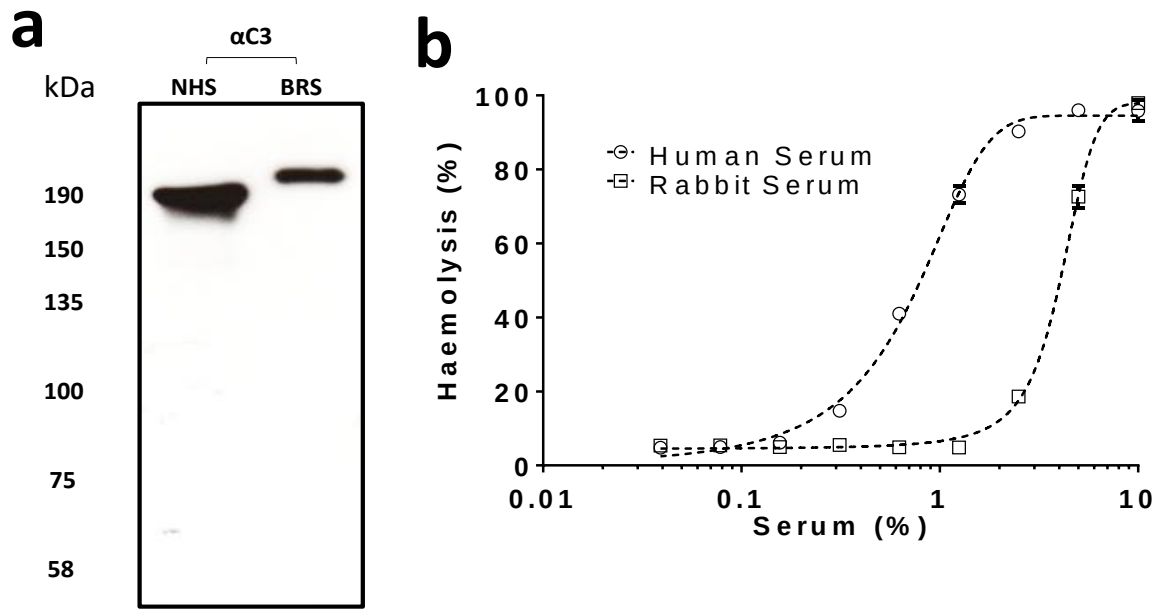


Figure 5. 1 Anti-C3 Western Blot and Haemolytic Assay with Human and Rabbit Serum
Differences in the activation of human and rabbit complement by a number of purified antibody subclasses was measured by ELISA. Human and rabbit C3 deposition was detected as a marker of complement activation following the incubation of human or rabbit serum on ELISA plates coated with a number of purified antibody subclasses. Human and rabbit C3 deposition was detected using a sheep anti-human C3/C3c antibody. ***a***, the cross-reactivity of the sheep anti-human C3/C3c antibody to human and rabbit C3 was assessed by western blot. 10µL of human (NHS) or rabbit (BRS) serum (diluted 1 in 400) was run through a 12.5% polyacrylamide gel under conditions, transferred to a nitrocellulose membrane and probed with the sheep anti-human C3/C3c antibody. A single C3-positive band was detected with both human and rabbit serum above the 190kDa marker proving the cross-reactivity of the sheep anti-human C3/C3c antibody to both human and rabbit C3. Rabbit C3 had a greater apparent molecular weight than human C3. ***b***, The complement activity of the human and rabbit serum used in subsequent functional assays was assessed by classical pathway haemolytic assay as describe in ***Section 2.8.1***. A range of human and rabbit serum concentrations (10-0.04%), water only (positive) or buffer only (negative) was incubated with antibody sensitised sheep erythrocytes in duplicate. Subsequent haemolysis (405nm) at each concentration of rabbit and human serum was measured and percentage haemolysis calculated. Each point represents the average percentage haemolysis achieved following incubation of erythrocytes with each concentration of human (open circles) or rabbit (open squares) serum. The error bars represent standard deviation. The dotted lines represent the non-linear regression model fit to each data set (sigmoidal, 4PL standard curve). It was concluded that both the human and rabbit serum were suitable sources of complement for use in subsequent functional assays.

5.2.1.1 Complement Deposition ELISA Development

An ELISA was set up to assess the optimal concentration of human and rabbit serum for use in the Human and Rabbit Complement Deposition ELISA (***Figure 5.2***). A range of concentrations of human or rabbit serum (10-0.001%) in the presence or absence of 0.01M EDTA (to inhibit complement activity) was incubated in duplicate on ELISA wells coated with 10µg/mL non-specific human IgG3. Human IgG3 was chosen due its superior ability to activate complement compared to the other subclasses (Bindon et al., 1988). The non-specific human IgG3 was affinity purified from human plasma was described in ***Section 2.2.4***. Subsequent human and rabbit C3 deposition (absorbance 492nm) was then detected with a sheep anti-human C3/C3c antibody. The concentration of human and rabbit serum resulting in maximum absorbance and 50% maximum absorbance was interpolated by fitting the data with a non-linear regression; sigmoidal, 4PL standard curve. The concentration of human serum resulting in maximum absorbance and 50% maximum absorbance was 0.48% and 0.19%, respectively (***Figure 5.2b***). The concentration of rabbit serum resulting in maximum absorbance and 50% maximum absorbance was 8.5% and 0.79%, respectively (***Figure 5.2a***). Minimal C3 deposition was detected in the presence of 0.01M EDTA. The optimal concentration of human and rabbit serum for use in the Human and Rabbit Complement Deposition ELISA was decided as 0.5% and 10%, respectively.

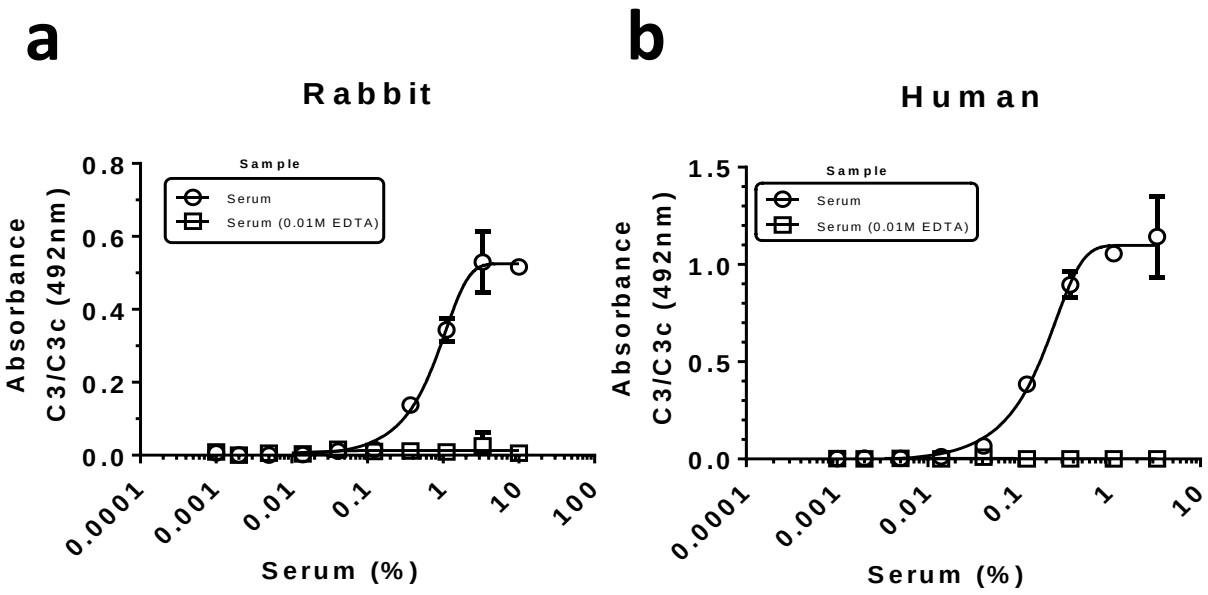


Figure 5. 2 Complement Activation of a Titration of Human and Rabbit Serum with Solid-Phase IgG3

Differences in the activation of human and rabbit complement by a number of purified antibody subclasses was measured by ELISA. Human and rabbit C3 deposition was detected as a marker of complement activation following the incubation of human or rabbit serum on ELISA plates coated with a number of purified antibody subclasses. Human and rabbit C3 deposition was detected using a sheep anti-human C3/C3c antibody. Firstly, an assay was set up to assess the suitable concentration of human (***b***) and rabbit (***a***) serum to be used in subsequent functional ELISAs. A range of concentrations of human or rabbit serum (10-0.001%) in the presence (open squares) or absence (open circles) of 0.01M EDTA (to inhibit complement activity) was incubated in duplicate on ELISA wells coated with 10µg/mL human IgG3. Human IgG3 was chosen due its superior ability to activate complement compared to the other subclasses (Bindon et al., 1988). Subsequent human and rabbit C3 deposition (absorbance 492nm) was then detected with a sheep anti-human C3/C3c antibody. Each point represents the mean average absorbance achieved with each antibody subclass in the presence of either human (***b***) or rabbit serum (***a***). The error bars represent the standard deviation. The concentration of human and rabbit serum resulting in maximum absorbance and 50% maximum absorbance was interpolated by fitting the data with a non-linear regression; sigmoidal, 4PL standard curve. The concentration of human serum resulting in maximum absorbance and 50% maximum absorbance was 0.48% and 0.19%, respectively. The concentration of rabbit serum resulting in maximum absorbance and 50% maximum absorbance was 8.5% and 0.79%, respectively. Minimal C3 deposition was detected in the presence of 0.01M EDTA.

An ELISA was set up to assess the optimal coating concentration of purified human IgG3 for use in the Human and Rabbit Complement Deposition ELISA (***Figure 5.3***). ELISA plates were coated in a range of concentrations human IgG3 (100-0.002µg/mL) and incubated with either human serum or rabbit serum in the presence or absence of 0.01M EDTA (to inhibit complement activity) in duplicate. Subsequent human and rabbit C3 deposition (absorbance 492nm) was then detected with a sheep anti-human C3/C3c antibody. The coating concentration of human IgG3 resulting in maximum absorbance and 50% maximum absorbance was interpolated by fitting the data with a non-linear regression; sigmoidal, 4PL standard curve. The coating concentration of human IgG3 resulting in maximum rabbit C3 absorbance and 50% maximum rabbit C3 absorbance was 8.5µg/mL and 2.9µg/mL, respectively (***Figure 5.3a***). The coating concentration of human IgG3 resulting in maximum human C3 absorbance and 50% maximum human C3 absorbance was 3.3µg/mL and 1.3µg/mL, respectively (***Figure 5.3b***). Minimal C3 deposition was detected in the presence of 0.01M EDTA. The optimal coating concentration of antibody for use in the Human and Rabbit Complement Deposition ELISA was decided 10µg/mL.

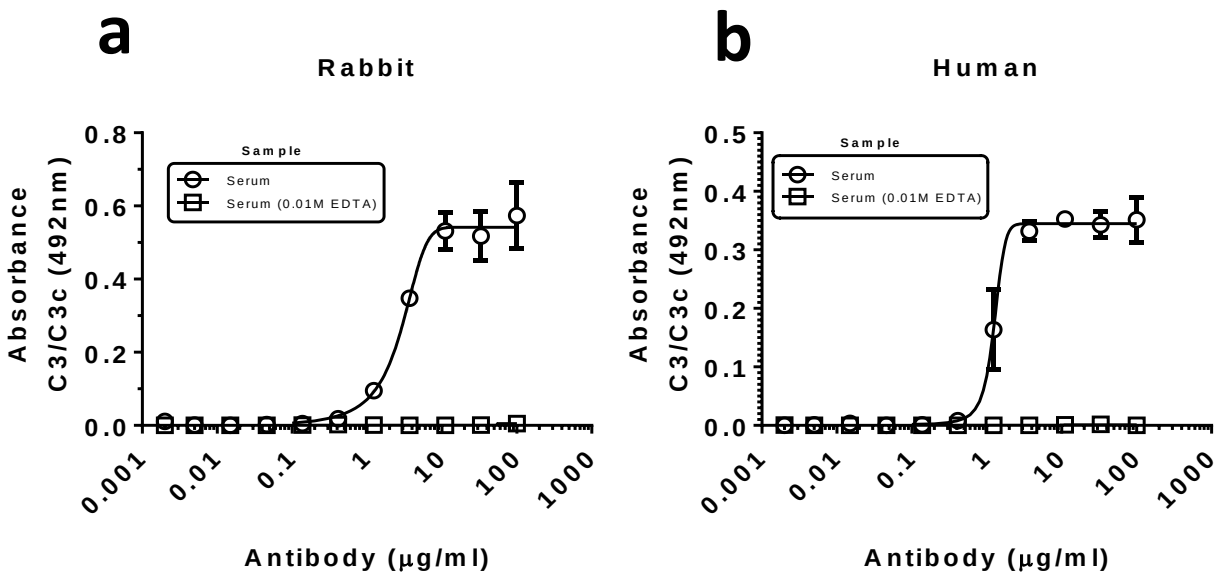


Figure 5. 3 Human and Rabbit Complement Activation with a Titration of Solid-Phase IgG3

Differences in the activation of human and rabbit complement by a number of purified antibody subclasses was measured by ELISA. Human and rabbit C3 deposition was detected as a marker of complement activation following the incubation of human or rabbit serum on ELISA plates coated with a number of purified antibody subclasses. Human and rabbit C3 deposition was detected using a sheep anti-human C3/C3c antibody. Firstly, an assay was set up to assess the suitable ELISA well coating concentration of human antibody subclasses to be used in subsequent functional ELISAs. ELISA plates were coated in a range of concentrations human IgG3 (100-0.002µg/mL) and incubated with either human serum (0.5%; ***b***) or rabbit serum (10%; ***a***) in the presence (open squares) or absence (open circles) of 0.01M EDTA (to inhibit complement activity) in duplicate. Human IgG3 was chosen due its superior ability to activate complement compared to the other subclasses (Bindon et al., 1988). Subsequent human and rabbit C3 deposition (absorbance 492nm) was then detected with a sheep anti-human C3/C3c antibody. Each point represents the mean average absorbance achieved with each coating concentration of human IgG in the presence of either human (***b***) or rabbit serum (***a***). The error bars represent the standard deviation. The coating concentration of human IgG3 resulting in maximum absorbance and 50% maximum absorbance was interpolated by fitting the data with a non-linear regression; sigmoidal, 4PL standard curve. ***a***, The coating concentration of human IgG3 resulting in maximum rabbit C3 absorbance and 50% maximum rabbit C3 absorbance was 8.5µg/mL and 2.9µg/mL, respectively. ***b***, The coating concentration of human IgG3 resulting in maximum human C3 absorbance and 50% maximum human C3 absorbance was 3.3µg/mL and 1.3µg/mL, respectively. Minimal C3 deposition was detected in the presence of 0.01M EDTA.

5.2.1.2 Human and Rabbit Complement Activation by Immunoglobulins

The ability of non-specific human IgG1, IgG2, IgG3, IgG4, IgM, human IgG, MenACWY-specific antibody and non-specific rabbit IgG (one subclass only) antibody to activate human and rabbit complement was assessed by ELISA (***Figure 5. 4***). Non-specific human IgG1, IgG2, IgG3, IgG4, IgM, human IgG and rabbit IgG was affinity purified from the pooled plasmas of three (human) and one (rabbit) individuals as described in ***Section 2.2.4*** and ***Section 2.2.2***. Human MenACWY-specific antibody was affinity purified from the pooled plasma of 14 adult individuals previously vaccinated with Mencevax ™ (plain polysaccharides from meningococcal serogroups A, C, W-135 and Y) as described in ***Section 2.2.3***. The antibody subclass composition of human IgG and anti-MenACWY antibody preparations was previously determined as described in ***Chapter 4: Figure 4.8***. Human serum (0.5%), rabbit serum (10%) or buffer only (assay blank) was incubated in duplicate on wells or ELISA wells coated with 10µg/mL of each antibody subclass preparation. Subsequent human and rabbit C3 deposition (absorbance 492nm) was then detected with a sheep anti-human C3/C3c antibody. The absorbance achieved following incubation of serum (minus blank) with each antibody preparation was compared by t-test statistical analysis. In the presence of human serum, the rank order of complement activation was IgG3>> IgG1>>>> IgM; IgG2; IgG4 (***Figure 5.4a***). In the presence of rabbit serum, the rank order of complement activation was IgG3>>> IgM; IgG1; IgG2> IgG4 (***Figure 5.4b***). These data show that human IgM is a more potent activator of rabbit complement that it is for human complement.

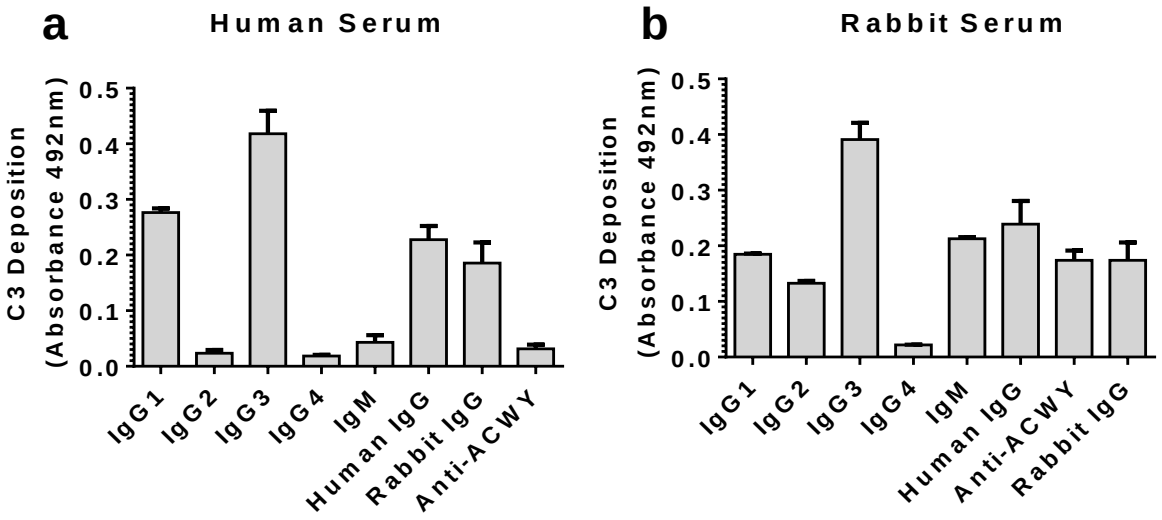


Figure 5. 4 Human and Rabbit Complement Activation by Antibody Subclasses
Differences in the activation of human and rabbit complement by a number of purified antibody subclasses was measured by ELISA. Human IgG1, IgG2, IgG3, IgG4, IgM, non-specific IgG and rabbit IgG antibodies were purified from plasma by affinity chromatography (as detailed in ***Section 2.2.2*** and ***Section 2.2.4***). The meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific antibody was affinity-purified from the pooled plasma of fourteen adult individuals taken at least one month post-vaccination with a quadrivalent plain polysaccharide (serogroups A, C, W-135 and Y) meningococcal vaccine (as detailed in ***Section 2.2.3***). The antibody subclass compositions of the non-specific human IgG and meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific antibody preparation are detailed in ***Figure 4.8***. Human serum (0.5%; ***a***), rabbit serum (10%; ***b***) or buffer only (blank) was incubated in duplicate on wells or ELISA wells coated with 10µg/mL of each antibody subclass preparation. Subsequent human and rabbit C3 deposition (absorbance 492nm) was then detected with a sheep anti-human C3/C3c antibody. Each point represents the mean average absorbance (minus the mean average absorbance of each blank) achieved with each antibody subclass in the presence of either human (***a***) or rabbit serum (***b***). The error bars represent the standard deviation. The absorbance achieved following incubation of serum (minus blank) with each antibody preparation was compared by t-test statistical analysis. ***a***, In the presence of human serum, the rank order of complement activation was IgG3>> IgG1>>>> IgM; IgG2; IgG4. ***b***, In the presence of rabbit serum, the rank order of complement activation was IgG3>>> IgM; IgG1; IgG2> IgG4.

5.2.2 Anti-Meningococcal Polysaccharide Complement Deposition Assay

In the Human and Rabbit Complement Deposition ELISA, antibody subclasses were directly coated to ELISA plates. As these antibodies are not bound to their antigen, they will not be arranged in a manner conducive to activating complement. To address this, an assay was designed to capture antigen-specific antibody to ELISA plates coated with antigen; thereby simulating the mechanism of complement activation in vivo. Briefly, purified meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific IgG1, IgG2 and IgM antibodies were captured onto ELISA plates coated with tetanus toxoid-conjugated meningococcal polysaccharides from serogroups A, C, W-135 and Y. Polysaccharide-specific antibody subclasses IgG3 and IgG4 could not be isolated in sufficient quantities for this and subsequent assays. C3 deposition was detected as a marker of activation after incubation of human and rabbit serum with each polysaccharide-bound antibody preparation.

5.2.2.1 Isolation of Anti-MenACWY IgG1, IgG2 and IgM Antibody

In preparation for the Anti-Meningococcal Polysaccharide Complement Deposition Assay, meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific antibody was affinity-purified from the pooled plasma of fourteen adult individuals (as detailed in ***Section 2.2.3***). Plasma samples were taken at least one month post-vaccination with a quadrivalent plain polysaccharide (serogroups A, C, W-135 and Y) meningococcal vaccine (Mencevax™). The purified meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific antibody was separated by antibody subclass by affinity chromatography by injection of the purified antibody over a series of columns packed with Sepharose conjugated to antibody subclass-specific proteins (as detailed in ***Section 2.2.2*** and ***Section 2.2.4***). The purity and composition of each purified antibody was assessed by SDS-PAGE (***Figure 5.5***). Each antibody preparation was run though a 10% polyacrylamide gel under reducing conditions and stained with coomassie Brilliant

Blue dye (***Figure 5.5a***). No contaminating proteins could be detected in any antibody preparation. By western blot, antibody subclass preparation was assessed for contamination with other antibody subclasses (***Figure 5.5b-d***). Each antibody preparation was run through three 10% polyacrylamide gels under reducing conditions. Once run, gels were transferred onto nitrocellulose membrane and probed with either a rabbit anti-human IgG1 (***Figure 5.5b***), a rabbit anti-human IgG2 (***Figure 5.5c***) or a donkey anti-human IgM (***Figure 5.5d***) antibody. It was concluded that there was no detectable contamination of each meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific antibody subclass preparation with any other of the antibody subclasses tested.

5.2.2.2 Preparation and Assessment of Anti-MenACWY Antibody-Depleted Human Serum

A source of human serum with minimal antibody specific to meningococcal polysaccharides from serogroups A, C, W and Y is required for the Anti-Meningococcal Polysaccharide Complement Deposition Assay. To produce a suitable source of human complement for these assays, human serum was depleted of meningococcal polysaccharide-specific antibody by injection over a TT-conjugated meningococcal polysaccharide (serogroups A, C, W-135 and Y) conjugated Sepharose column as described in ***Section 2.5.4.1***.

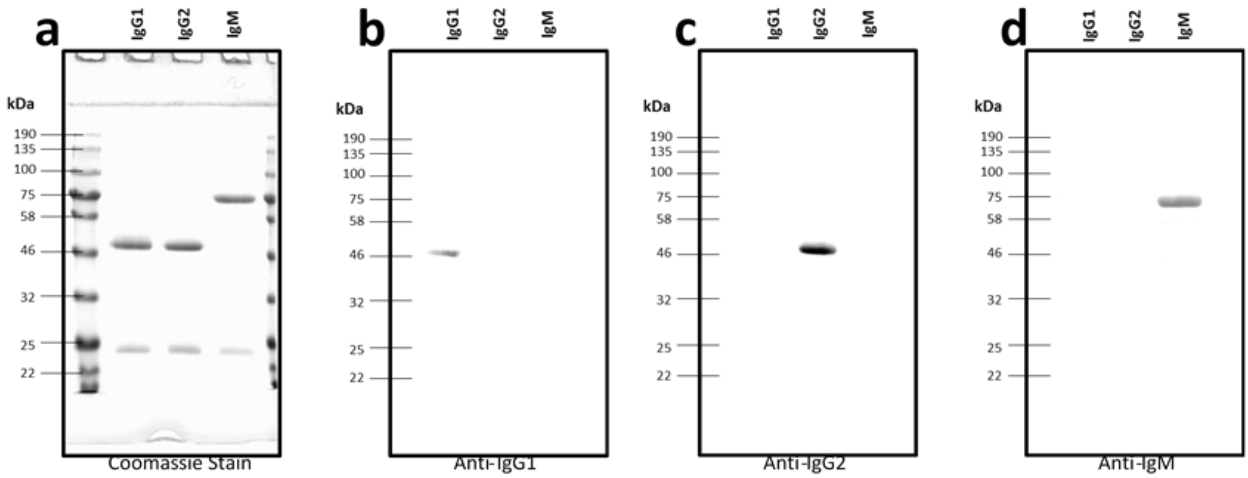


Figure 5. 5 SDS-PAGE Analysis of Anti-MenACWY IgG1, IgG2 and IgM Antibodies
Meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific antibody was affinity-purified from the pooled plasma of fourteen adult individuals (as detailed in ***Section 2.2.3***). Plasma samples were taken at least one month post-vaccination with a quadrivalent plain polysaccharide (serogroups A, C, W-135 and Y) meningococcal vaccine (Mencevax™). The purified meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific antibody was separated by antibody subclass by affinity chromatography by injection of the purified antibody over a series of columns packed with Sepharose conjugated to antibody subclass-specific proteins (as detailed in ***Section 2.2.2*** and ***Section 2.2.4***). The purity and composition of each purified antibody was assessed by SDS-PAGE. ***a***, 200ng of each antibody preparation was run though a 10% polyacrylamide gel under reducing conditions and stained with coomassie Brilliant Blue dye. No contaminating proteins could be detected in any antibody preparation. By western blot, antibody subclass preparation was assessed for contamination with other antibody subclasses. 200ng of each antibody preparation was run through three 10% polyacrylamide gels under reducing conditions. Once run, gels were transferred onto nitrocellulose membrane and probed with either a rabbit anti-human IgG1 (blot ***b***), a rabbit anti-human IgG2 (blot ***c***) or a donkey anti-human IgM (blot ***d***) antibody (Stratech). In blot ***b*** (anti-human IgG1), a positive band was detected in the lane containing purified human IgG1 only. In blot ***c*** (anti-human IgG2), a positive band was detected in the lane containing purified human IgG2 only. In blot ***d*** (anti-human IgM), a positive band was detected in the lane containing purified human IgM only. These data confirmed that there was no detectable contamination of each meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific antibody subclass preparation with any other of the antibody subclasses tested.

The reactivity of human serum and the meningococcal polysaccharide-specific antibody-depleted human serum was assessed by ELISA (***Figure 5.6a***). A titration of serum was incubated in duplicate on an ELISA plate coated with meningococcal polysaccharides from serogroups A, C, W-135 and Y. Subsequent IgG binding was detected using a donkey anti-human IgG antibody. By two-way ANOVA multiple comparison significantly more IgG binding was detected with human serum compared to the meningococcal polysaccharide-specific antibody-depleted human serum confirming efficient depletion of meningococcal polysaccharide-specific antibody.

To ensure anti-MenACWY antibody depletion of human serum had not altered complement activity, the lytic activity of non-depleted and anti-MenACWY depleted human serum was assessed by classical pathway haemolytic assay (***Figure 5. 6b***). Haemolysis of sensitised sheep erythrocytes (2%) was measured following a thirty-minute incubation with 10-0.26% non-depleted and depleted serum. Two-way ANOVA statistical analysis showed that there was no significant difference in haemolytic activity between human serum and meningococcal polysaccharide-specific antibody-depleted human serum.

An assay was set up to check whether depletion of anti-MenACWY-TT antibody had successfully reduced endogenous complement activity of human serum with MenACWY-TT antigens. C3 deposition, following incubation of a titration (20-0.3%) of unmodified human serum, anti-MenACWY-TT depleted serum and rabbit serum with MenACWY-TT antigens, was measured by ELISA (***Figure 5. 6c***). By two-way ANOVA multiple comparison significantly more C3 deposition was detected with human serum compared to rabbit serum and the meningococcal polysaccharide-specific antibody-depleted human serum confirming efficient depletion of meningococcal polysaccharide-specific antibody. From these assays, it was concluded that human serum was successfully depleted of the exogenous meningococcal polysaccharide specific antibody without altering complement activity and thus was an acceptable source of complement for the Anti-Meningococcal Polysaccharide Complement Deposition Assay.

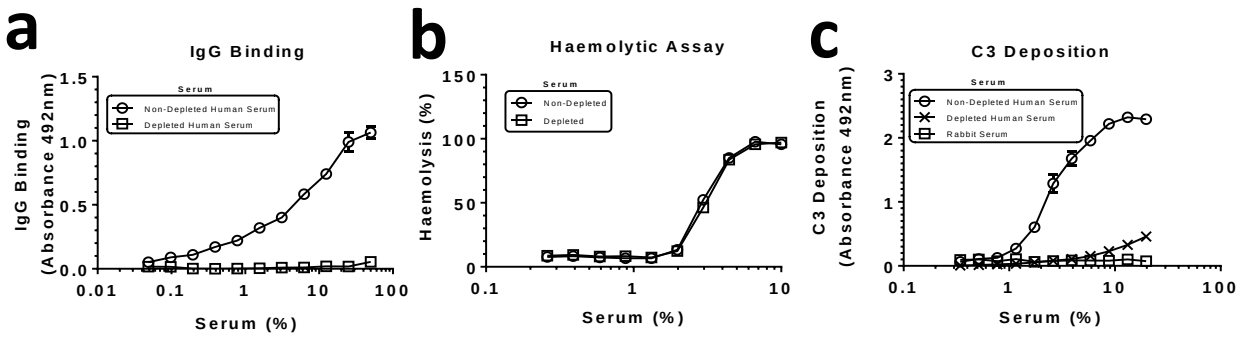


Figure 5. 6 Assessment of Anti-MenACWY Antibody-Depleted Human Serum

a, Differences in the activation of human and rabbit complement by antigen-bound human antibody subclasses was measured by ELISA as described in ***Figure 5.7***. A source of human serum with minimal antibody specific to meningococcal polysaccharides from serogroups A, C, W and Y is required for these assays. To produce a suitable source of human complement for these assay, human serum was depleted of meningococcal polysaccharide-specific antibody by injection over a TT-conjugated meningococcal polysaccharide (serogroups A, C, W-135 and Y) conjugated Sepharose column as described in ***Section 2.5.4.1***. ***a***, The reactivity of human serum (open circles) and the meningococcal polysaccharide-specific antibody-depleted human serum (open squares) was assessed by ELISA. A titration of serum was incubated in duplicate on an ELISA plate coated with meningococcal polysaccharides from serogroups A, C, W-135 and Y. Subsequent IgG binding (absorbance 492nm) was detected using a donkey anti-human IgG antibody. Each point represents the mean average absorbance achieved as each dilution of serum. The error bars represent the standard deviation. By two-way ANOVA multiple comparison significantly more IgG binding was detected with human serum compared to the meningococcal polysaccharide-specific antibody-depleted human serum confirming efficient depletion of meningococcal polysaccharide-specific antibody. ***b***, To ensure meningococcal polysaccharide-specific antibody-depletion of human serum had not altered complement activity, the complement activity of human serum and meningococcal polysaccharide-specific antibody-depleted human serum was assessed by haemolytic assay as described in ***Section 2.8.1***. A range of serum concentrations (10-0.25%) was incubated with antibody sensitised sheep erythrocytes in duplicate. Subsequent haemolysis at each concentration serum was measured and percentage haemolysis calculated. Each point represents the average percentage haemolysis achieved following incubation of erythrocytes with each concentration of serum. Two-way ANOVA statistical analysis showed that there was no significant difference in haemolytic activity between human serum and meningococcal polysaccharide-specific antibody-depleted human serum. ***c***, Complement activation of human serum (open circles), the meningococcal polysaccharide-specific antibody-depleted human serum (crosses) and rabbit serum (open squares) by meningococcal polysaccharide was assessed by ELISA. A titration of serum was incubated in duplicate on an ELISA plate coated with meningococcal polysaccharides from serogroups A, C, W-135 and Y. Subsequent C3 deposition (absorbance 492nm) was detected using a goat anti-human C3/C3c antibody. Each point represents the mean average absorbance achieved as each dilution of serum. The error bars represent the standard deviation. By two-way ANOVA multiple comparison significantly more C3 deposition was detected with human serum compared to rabbit serum and the meningococcal polysaccharide-specific antibody-depleted human serum confirming efficient depletion of meningococcal polysaccharide-specific antibody.

5.2.2.3 Human and Rabbit Complement Activation by Antigen-bound Immunoglobulins

Differences in the activation of human and rabbit complement by antigen-bound human antibody subclasses was measured by ELISA (***Figure 5.7***). Purified meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific IgG1, IgG2 and IgM antibodies were incubated in duplicate at a range of concentration (40-0.04µg/mL) on ELISA plates coated with 20µg/mL tetanus toxoid-conjugated meningococcal polysaccharides from serogroups A, C, W-135 and Y. Meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific IgG1, IgG2 and IgM antibodies were affinity-purified from the pooled plasma of fourteen adult individuals (as detailed in ***Figure 5.5***). Tetanus toxoid-conjugated meningococcal polysaccharide-specific antibody-depleted human serum (***Figure5.7a***; 0.5%) or rabbit serum (***Figure5.7b***; 10%) was then added in duplicate to each concentration of antigen-bound meningococcal polysaccharide specific IgG1, IgG2 and IgM antibodies. Subsequent human and rabbit C3 deposition (absorbance 492nm) was then detected with a sheep anti-human C3/C3c antibody. By two-way ANOVA multiple comparison statistical analysis comparing human C3 deposition with each antibody subclass, the rank order of activation was IgG1>>> IgM> IgG2. By two-way ANOVA multiple comparison statistical analysis comparing rabbit C3 deposition with each antibody subclass, the rank order of activation was IgM> IgG1>> IgG2. These data show that human IgM is a more potent activator of rabbit complement that it is for human complement.

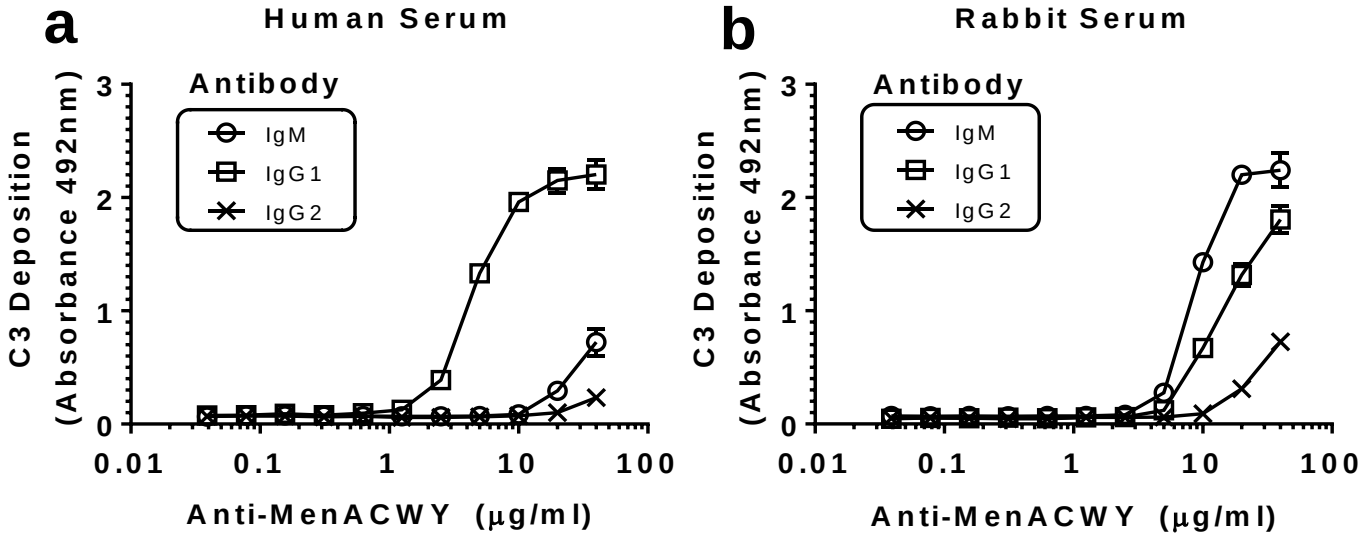


Figure 5. 7 Human and Rabbit Complement Activation by MenACWY-bound IgG1, IgG2 and IgM Antibodies

Differences in the activation of human and rabbit complement by antigen-bound human antibody subclasses was measured by ELISA. Purified meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific IgG1 (open squares), IgG2 (crosses) and IgM (open circles) antibodies were incubated in duplicate at a range of concentration (40-0.04µg/mL) on ELISA plates coated with 20µg/mL tetanus toxoid-conjugated meningococcal polysaccharides from serogroups A, C, W-135 and Y. Meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific IgG1, IgG2 and IgM antibodies were affinity-purified from the pooled plasma of fourteen adult individuals (as detailed in ***Figure 5.5***). Tetanus toxoid-conjugated meningococcal polysaccharide-specific antibody-depleted human serum (***a***; 0.5%) or rabbit serum (***b***; 10%) was then added in duplicate to each concentration of antigen-bound meningococcal polysaccharide specific IgG1, IgG2 and IgM antibodies. Subsequent human and rabbit C3 deposition (absorbance 492nm) was then detected with a sheep anti-human C3/C3c antibody. Each point represents the mean average absorbance achieved with each antigen-bound antibody subclass in the presence of either human (***a***) or rabbit serum (***b***). The error bars represent the standard deviation. By two-way ANOVA multiple comparison statistical analysis comparing human C3 deposition with each antibody subclass, the rank order of activation was IgG1>>> IgM> IgG2. By two-way ANOVA multiple comparison statistical analysis comparing rabbit C3 deposition with each antibody subclass, the rank order of activation was IgM> IgG1>> IgG2.

5.2.3 Complement Activation by Anti-MenACWY Antibody Subclasses Bound to Neisseria Meningitidis serogroup W-135 (strain 102/98)

The ability of anti-MenACWY IgG1, IgG2 and IgM antibodies to activate human complement when bound to *Neisseria meningitidis* serogroup W-135 (strain 102/98) bacteria was assessed by SBA and complement deposition assay as described in ***Section 2.9*** and ***Section 2.10.3***. The IgG-depleted serum was prepared as described by Brookes et al., (2013). Only one serogroup of bacteria was used in these assays due to time restraints. *Neisseria meningitidis* serogroup W-135 (strain 102/98) was chosen as the focus in the following and subsequent assays due to its current relevance. The global incidence of invasive meningococcal disease caused by serogroup W-135 has significantly increased in the past 5 years (Ladhani et al., 2015; Mustapha et al., 2016).

Firstly, binding of the anti-MenACWY IgG1, IgG2 and IgM antibodies to *Neisseria meningitidis* serogroup W-135 (strain 102/98) bacteria was assessed by flow cytometry (***Figure 5.8***). Meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific IgG1, IgG2 and IgM antibodies were affinity-purified from the pooled plasma of fourteen adult individuals (as detailed in ***Figure 5.5***). A serial dilution of each antibody preparation (diluted to 250-3.9µg/mL) was incubated with *Neisseria Meningitidis* serogroup W-135 (strain 102/98) in duplicate. The bacteria were also incubated with either buffer only (negative control; -ve) or heat-inactivated mouse anti-meningococcal polysaccharide (serogroups A, C, W-135 and Y) serum (positive control; +ve). The positive control serum was obtained from a mouse previously immunised with the plain meningococcal polysaccharides of serogroups A, C, W-135 and Y. Subsequent IgG1, IgG2 and IgM binding (fluorescence) to *Neisseria Meningitidis* serogroup W-135 (strain 102/98) was detected with a goat anti-human IgG/IgM FITC conjugated antibody (Jackson ImmunoResearch). It was concluded that each of the purified meningococcal polysaccharide-specific antibodies were able to bind to *Neisseria Meningitidis* serogroup W-135 (strain 102/98).

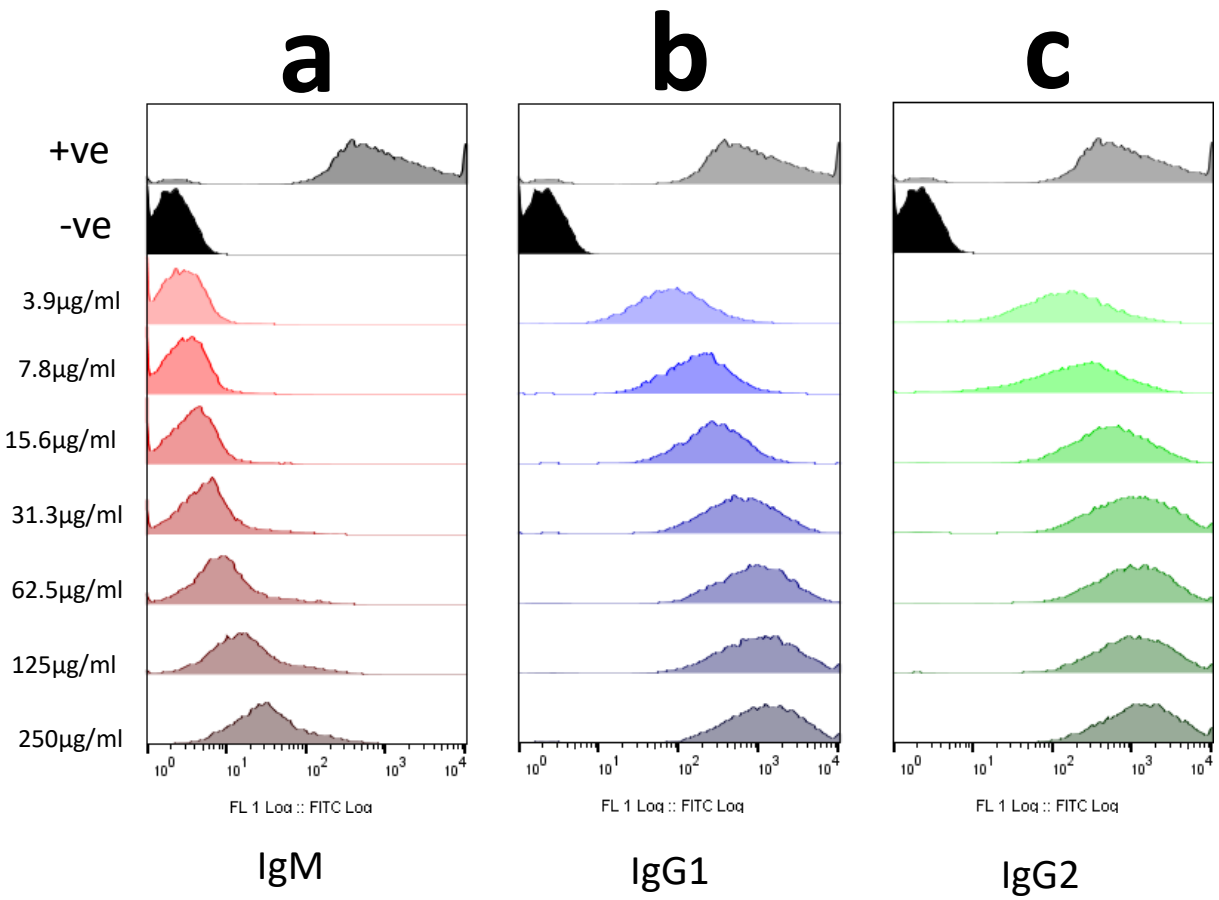


Figure 5. 8 Anti-MenACWY IgG1, IgG2 and IgM Antibody Binding to *Neisseria Meningitidis* Serogroup W (strain 102/98)

Meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific IgG1, IgG2 and IgM antibodies were affinity-purified from the pooled plasma of fourteen adult individuals (as detailed in ***Figure 5.5***). Plasma samples were taken at least one month post-vaccination with a quadrivalent plain polysaccharide (serogroups A, C, W-135 and Y) meningococcal vaccine (Mencevax™). Binding of the purified meningococcal polysaccharide-specific IgM (***a***), IgG1 (***b***) and IgG2 (***c***) antibodies was assessed by flow cytometry. A serial dilution of each antibody preparation (diluted to 250-3.9µg/mL) was incubated with *Neisseria Meningitidis* serogroup W-135 (strain 102/98) in duplicate. The bacteria were also incubated with either buffer only (negative control; -ve) or heat-inactivated mouse anti-meningococcal polysaccharide (serogroups A, C, W-135 and Y) serum (positive control; +ve). The positive control serum was obtained from a mouse previously immunised with the plain meningococcal polysaccharides of serogroups A, C, W-135 and Y. Subsequent IgG1, IgG2 and IgM binding (fluorescence) to *Neisseria Meningitidis* serogroup W-135 (strain 102/98) was detected with a goat anti-human IgG/IgM FITC conjugated antibody (Jackson ImmunoResearch). Each histogram represents the fluorescence achieved following incubation of the bacteria with each concentration of purified meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific IgM (***a***), IgG1 (***b***) and IgG2 (***c***) antibody and positive and negative controls. The fluorescence of 10,000 bacteria for each sample was analysed in the fluorescence channel. It was concluded that each of the purified meningococcal polysaccharide-specific antibodies were able to bind to *Neisseria Meningitidis* serogroup W-135 (strain 102/98).

After confirming that purified meningococcal polysaccharide-specific antibody subclasses could bind *Neisseria meningitidis* serogroup W-135 (strain 102/98) bacteria, survival of *Neisseria meningitidis* serogroup W bacteria, incubated with 25% IgG-depleted human serum in the presence or absence of the purified anti-MenACWY antibody subclasses, was assessed by SBA (***Figure 5. 9a***). Meningococcal polysaccharide-specific IgG1 (7.8µg/ml), IgG2 (7.8µg/ml) and IgM (500µg/ml) antibodies were incubated with live *Neisseria Meningitidis* serogroup W-135 (strain 102/98) in duplicate. The bacteria were also incubated with either buffer only (serum and HI serum) or heat-inactivated mouse anti-meningococcal polysaccharide serum (positive). Once incubated, IgG-depleted human serum (25%; prepared as described by Brookes et al., 2013) or 25% heat-inactivated IgG-depleted human serum (HI serum) was added to each preparation of bacteria. Each preparation of bacteria was grown on a blood agar plate overnight and the number of colony forming units (CFU) was counted for each. By one-way ANOVA, no significant difference in bacterial growth was detected in the presence of 25% IgG-depleted human serum and purified anti-MenACWY IgG1 (7.8µg/ml), IgG2 (7.8µg/ml) or IgM (500µg/ml) antibody when compared to 25% IgG-depleted human serum alone. Significantly increased bacterial growth was detected when incubated with 25% IgG-depleted HI human serum alone ($P<0.0001$) and significantly reduced bacterial growth was detected when incubated with 25% IgG-depleted human serum and HI immune sera (1/2 dilution) ($P<0.001$) when compared to incubation with 25% IgG-depleted human serum alone. From these data it was concluded that the purified meningococcal polysaccharide-specific antibody subclasses had no bactericidal activity towards *Neisseria Meningitidis* serogroup W-135 (strain 102/98) bacteria.

The deposition of C3 on *Neisseria meningitidis* serogroup W-135 (strain 102/98) bacteria, incubated with 5% IgG-depleted human serum in the presence or absence of purified anti-MenACWY antibody subclasses, was assessed by flow cytometry (complement deposition assay; ***Figure 5. 9b***). Meningococcal polysaccharide-specific IgG1 (3.1µg/ml), IgG2 (3.1µg/ml) and IgM (200µg/ml) antibodies were incubated with killed *Neisseria Meningitidis* serogroup W-135 (strain 102/98). The bacteria were also incubated with either buffer only (serum and no serum) or heat-inactivated mouse anti-meningococcal polysaccharide serum (positive). Once incubated, IgG-depleted human serum (5%) or buffer (no serum) was added to each preparation of bacteria. Subsequent complement deposition (median fluorescence) was detected using a rabbit anti-human C3c FITC-conjugated antibody (Abcam).By one-way ANOVA, no significant difference in C3 deposition was detected in the presence of 5% IgG-depleted human serum and purified anti-MenACWY IgG1 (3.1µg/ml), IgG2 (3.1µg/ml) or IgM (200µg/ml) antibody when compared to 5% IgG-depleted human serum alone. Significantly increased C3 deposition was detected when incubated with 5% IgG-depleted human serum and HI immune serum (1/5 dilution) ($P<0.0001$) when compared to incubation with 5% IgG-depleted human serum alone. From these data it was concluded that the purified meningococcal polysaccharide-specific antibody subclasses could not induce complement deposition on *Neisseria Meningitidis* serogroup W-135 (strain 102/98) bacteria.

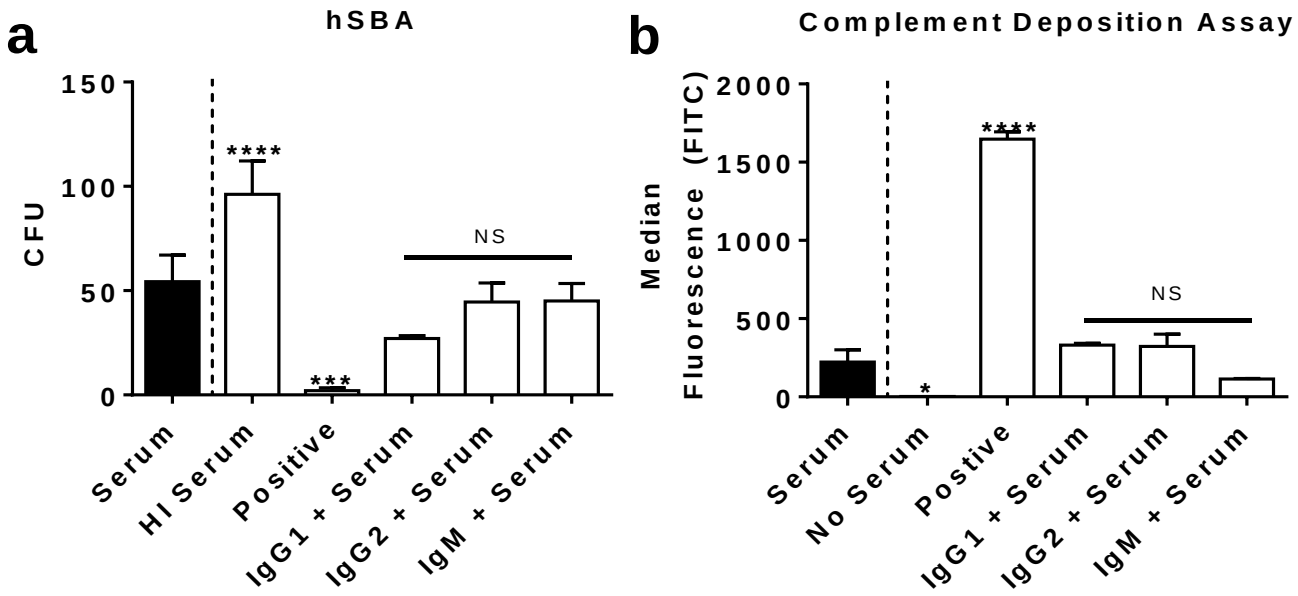


Figure 5. 9 Complement Activation by Anti-MenACWY Antibody Subclasses Bound to *Neisseria Meningitidis* Serogroup W-135 (strain 102/98)

The ability of anti-MenACWY IgG1, IgG2 and IgM antibodies to activate human complement when bound to *Neisseria meningitidis* serogroup W-135 (strain 102/98) bacteria was assessed by serum bactericidal assay (hSBA; ***a***) and flow cytometry (complement deposition assay; ***b***) Meningococcal polysaccharide (serogroups A, C, W-135 and Y) specific IgG1, IgG2 and IgM antibodies were affinity-purified from the pooled plasma of fourteen adult individuals (as detailed in ***Figure 5.5***). ***a***, Meningococcal polysaccharide-specific IgG1 (7.8µg/ml), IgG2 (7.8µg/ml) and IgM (500µg/ml) antibodies were incubated with live *Neisseria Meningitidis* serogroup W-135 (strain 102/98) in duplicate. The bacteria were also incubated with either buffer only (serum and HI serum) or heat-inactivated mouse anti-meningococcal polysaccharide serum (positive). Once incubated, IgG-depleted human serum (25%; prepared as described by Brookes et al., 2013) or 25% heat-inactivated IgG-depleted human serum (HI serum) was added to each preparation of bacteria. Each preparation of bacteria was grown on a blood agar plate overnight and the number of colony forming units (CFU) was counted for each. Each bar represents the average CFU achieved with each preparation and the error bars represent the standard deviation. The significant difference between the number of CFU for bacteria incubated with serum only and the other preparations was assessed by t-test statistical analysis. The asterisks indicate the statistical significance of the difference between the average CFU between complement deposition of bacteria incubated with serum only control and the other preparations (* = P value<0.05; *** = P value<0.001; **** = P value<0.0001; NS = not significant). ***b***, Meningococcal polysaccharide-specific IgG1 (3.1µg/ml), IgG2 (3.1µg/ml) and IgM (200µg/ml) antibodies were incubated with killed *Neisseria Meningitidis* serogroup W-135 (strain 102/98). The bacteria were also incubated with either buffer only (serum and no serum) or heat-inactivated mouse anti-meningococcal polysaccharide serum (positive). Once incubated, IgG-depleted human serum (5%) or buffer (no serum) was added to each preparation of bacteria. Subsequent complement deposition (median fluorescence) was detected using a rabbit anti-human C3c FITC-conjugated antibody (Abcam). Each bar represents the average median fluorescence achieved with each preparation and the error bars represent the standard deviation. The significant difference between complement deposition of bacteria incubated with serum only control and the other preparations was assessed by t-test statistical analysis.

5.3 Human and Rabbit Complement Pathways and *Neisseria Meningitidis*

The importance of each complement pathway in the clearance of *Neisseria meningitidis* with rabbit serum is poorly understood and it may be hypothesised that species-specific differences in the interaction of the complement activation pathways with *Neisseria meningitidis* contribute to the poor correlation between hSBA and rSBA titres. The activities of each pathway of complement activation in human and rabbit serum towards *Neisseria meningitidis* serogroup W-135 (strain 102/98) were compared using a series of pathway-specific inhibitors.

5.3.1 Assessment of Complement Inhibitors

5.3.1.1 Cross-Reactivity with Human and Rabbit Serum

To assess the activity of the classical, alternative and lectin pathways of human and rabbit complement towards *Neisseria meningitidis* serogroup W (strain 102/98), three pathway-specific blocking antibodies were incubated with either human or rabbit serum prior to incubating with *Neisseria meningitidis* serogroup W (strain 102/98) bacteria. The specificity of these antibodies was factor B (FB; alternative pathway blocking antibody; kindly gifted by Dr. Córdoba of the Centro de Investigaciones Biológicas, Madrid), C1q (Pathway Diagnostics) and MBL (R&D Systems). The cross-reactivity of these pathway-specific blocking antibodies to human and rabbit serum was assessed by western blot (***Figure 5.10***).

When probed with the blocking anti-FB antibody, chemiluminescence was detected in both human and rabbit serum proving species cross-reactivity of the anti-FB antibody (***Figure 5. 10a***). A single FB-positive band was detected in human serum at a molecular weight of 100kDa and at 84.4kDa in rabbit serum. When probed with the blocking anti-C1q antibody,

chemiluminescence was detected only with human serum (***Figure 5. 10b***). A single C1q-positive band was detected in human serum at a molecular weight of 49.3kDa corresponding to the a-b chain of human C1q. When probed with the blocking anti-MBL antibody, chemiluminescence was detected in both human and rabbit serum proving species cross-reactivity of the anti-MBL antibody (***Figure 5. 10c***). The predominant MBL-positive band detected in human and rabbit serum was at a molecular weight of 32kDa and at 31.4kDa, respectively.

5.3.1.2 Validation of Complement Inhibition

The concentration of an anti-C1q antibody required to block complement activation by the classical pathway was assessed by haemolytic assay (***Figure 5. 11***). The concentration of human (***Figure 5. 11a***) and rabbit (***Figure 5. 11c***) serum required for 80% haemolysis of sensitised sheep erythrocytes was calculated as 2.9% and 13.1%, respectively. Approximately, 15nM anti-C1q antibody was sufficient to block the haemolytic activity of 2.9% human serum (***Figure 5. 11b***). No inhibition of haemolytic activity of 13.1% rabbit serum was seen with the anti-C1q antibody at any concentration assessed (***Figure 5. 11d***).

The concentration of an anti-FB antibody required to block complement activation by the alternative pathway was assessed by haemolytic assay (***Figure 5. 12***). The concentration of 0.01M EGTA human serum required for 80% haemolysis of rabbit erythrocytes was calculated as 39.8% (***Figure 5. 12a***). Approximately, 300nM anti-FB antibody was sufficient to block the haemolytic activity of 39.8% human serum (***Figure 5. 12b***).

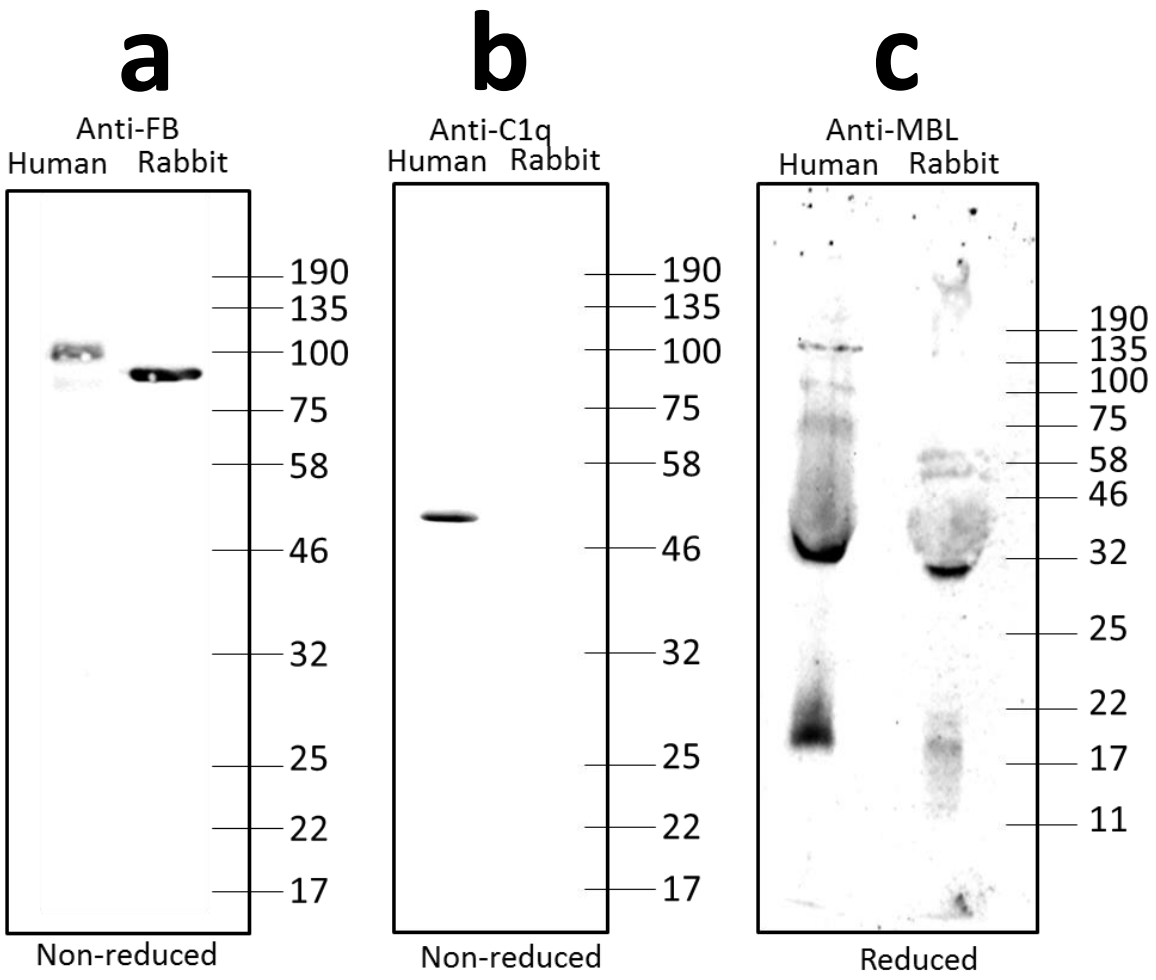


Figure 5. 10 Western Blot Analysis of Complement Pathway Inhibitors

To assess the activity of the classical, alternative and lectin pathways of human and rabbit complement towards *Neisseria meningitidis* serogroup W (strain 102/98), three pathway-specific blocking antibodies were incubated with either human or rabbit serum prior to incubating with *Neisseria meningitidis* serogroup W (strain 102/98) bacteria. The specificity of these antibodies was factor B (FB; alternative pathway blocking antibody; kindly gifted by Dr. Córdoba of the Centro de Investigaciones Biológicas, Madrid), C1q (Pathway Diagnostics) and MBL (R&D Systems). The cross-reactivity of these pathway-specific blocking antibodies to human and rabbit serum was assessed by western blot. 10µL of human or rabbit serum (diluted 1 in 400) was run through a 12.5% polyacrylamide gel under non-reducing (blots ***a*** and ***b***) or reducing conditions (blot ***c***), transferred to a nitrocellulose membrane and probed with either the mouse anti-human FB blocking antibody (blot ***a***), the mouse anti-human C1q blocking antibody (blot ***b***) or the mouse anti-human MBL blocking antibody (blot ***c***). Both the anti-FB blocking antibody and the anti-MBL blocking antibody were shown to be reactive to both human and rabbit FB and MBL, respectively. In contrast, the anti-C1q blocking antibody was shown to be reactive to human C1q only and did not bind rabbit C1q.

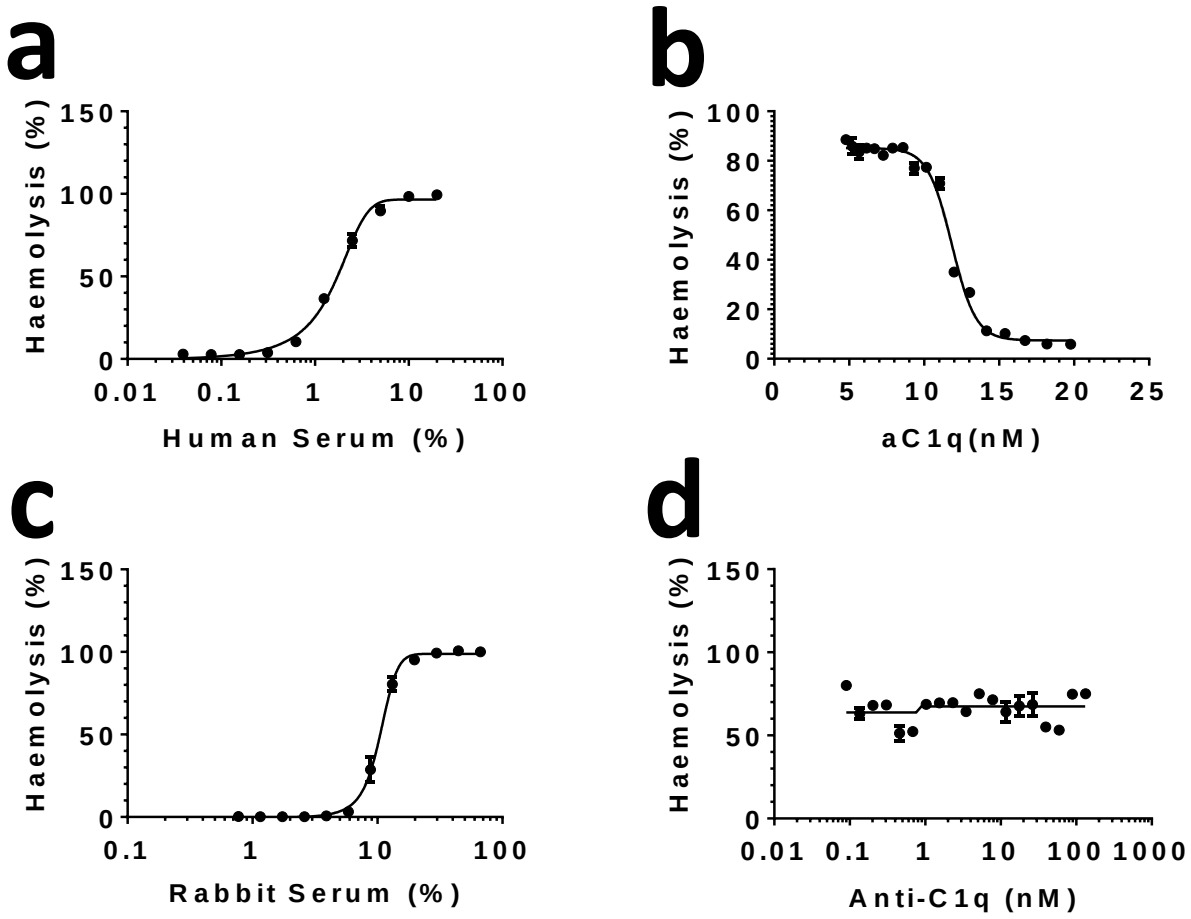


Figure 5. 11 Inhibition of Haemolytic Activity of Human and Rabbit Serum with an Anti-C1q Antibody

The activity of the classical, alternative and lectin pathways of human and rabbit complement towards *Neisseria meningitidis* serogroup W bacteria (strain 102/98) was assessed using three pathway-specific blocking antibodies. The concentration of the mouse anti-human C1q blocking antibody (Pathway Diagnostics) required to block the classical pathway of complement activation in human (***b***) and rabbit (***d***) serum was assessed by haemolysis assay as described in ***Section 2.8.1.2***. Firstly, the concentration of human and rabbit serum resulting in 80% haemolysis of antibody-sensitised sheep erythrocytes was assessed. A range of serum concentrations (10-0.25%) was incubated with antibody-sensitised sheep erythrocytes in duplicate. Subsequent haemolysis at each concentration human (***a***) or rabbit (***c***) serum was measured and percentage haemolysis calculated. Each point represents the average percentage haemolysis achieved following incubation of erythrocytes with each concentration of serum. The error bars represent the standard deviation. The concentration of human and rabbit serum resulting in 80% haemolysis was interpolated by fitting the data with a non-linear regression; sigmoidal, 4PL standard curve. The concentration of human and rabbit serum required for 80% haemolysis was calculated as 2.9% and 13.1%, respectively. Next, human (2.9%; ***b***) and rabbit (13.1%; ***d***) serum was incubated with range of concentrations of the mouse anti-human C1q blocking antibody followed by antibody-sensitised sheep erythrocytes in duplicate. Subsequent haemolysis at each concentration of the mouse anti-human C1q blocking antibody was measured and percentage haemolysis calculated. Each point represents the average percentage haemolysis achieved following incubation of erythrocytes with each human (***b***) and rabbit (***d***) serum pre-incubated with each concentration of the mouse anti-human C1q blocking antibody. The error bars represent the standard deviation.

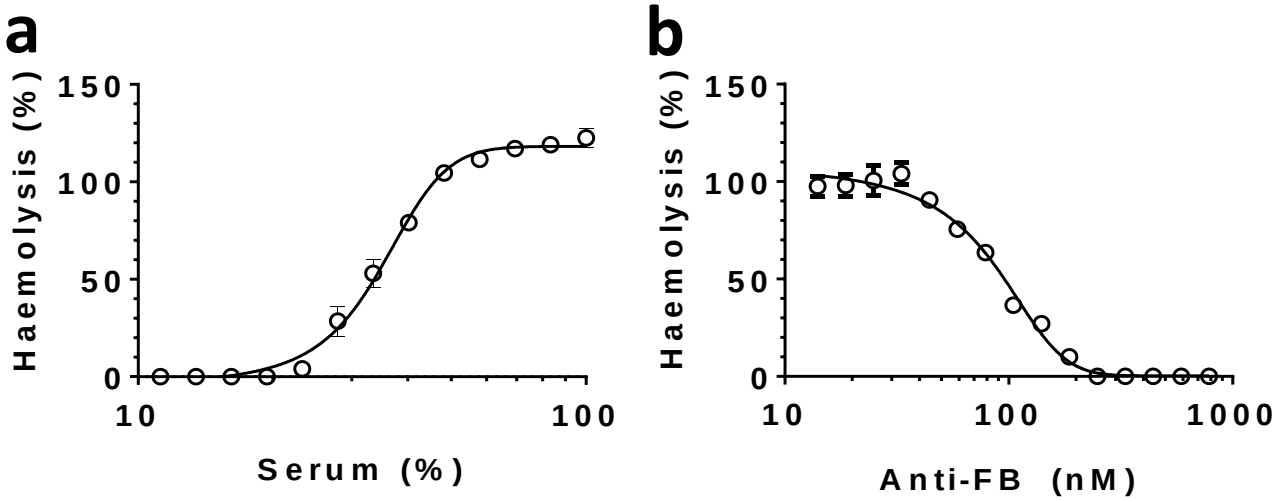


Figure 5. 12 Inhibition of Haemolytic Activity of Human Serum with an Anti-FB Antibody

The activity of the classical, alternative and lectin pathways of human and rabbit complement towards *Neisseria meningitidis* serogroup W bacteria (strain 102/98) was assessed using three pathway-specific blocking antibodies. The concentration of the mouse anti-human FB blocking antibody required to block the alternative pathway of complement activation in human serum was assessed by haemolysis assay as described in ***Section 2.8.2.1***. ***a***, Firstly, the concentration of human serum resulting in 80% haemolysis of rabbit erythrocytes (in the presence of 0.01M EGTA) was assessed. A range of serum concentrations (100-11%) was incubated with rabbit erythrocytes (in the presence of 0.01M EGTA) in duplicate. Each point represents the average percentage haemolysis achieved following incubation of erythrocytes with each concentration of serum. The error bars represent the standard deviation. The concentration of human serum resulting in 80% haemolysis was interpolated by fitting the data with a non-linear regression; sigmoidal, 4PL standard curve. The concentration of human serum required for 80% haemolysis was calculated as 39.8%. ***b***, Next, human (39.8%) serum was incubated with range of concentrations of the mouse anti-human FB blocking antibody followed by rabbit erythrocytes (in the presence of 0.01M EGTA) in duplicate. Subsequent haemolysis at each concentration of the mouse anti-human FB blocking antibody was measured and percentage haemolysis calculated. Each point represents the average percentage haemolysis achieved following incubation of erythrocytes with each human serum pre-incubated with each concentration of the mouse anti-human FB blocking antibody. The error bars represent the standard deviation. The mouse anti-human FB blocking antibody, at a concentration of 250nM, was sufficient to block the haemolytic activity of human serum.

The concentration of an anti-MBL antibody required to block complement activation by the lectin pathway was assessed by ELISA (***Figure 5. 13***). A titration of human serum was incubated on an ELISA plate coated with 200µg/mL mannan and subsequent C3 deposition detected (***Figure 5. 13a***). The concentration of human serum required for 80% C3 deposition was calculated as 2%. Human serum (2%) was incubated on an ELISA plates coated with 200µg/mL mannan in the presence of a titration of the anti-MBL antibody (***Figure 5. 13b***). Approximately, 200nM (30µg/mL) anti-MBL antibody was sufficient to block human lectin pathway complement activation.

The impact of 250nM mouse anti-human FB, 250nM mouse anti-human MBL and 15nM mouse anti-human C1q blocking antibodies as well as 1.94µM purified human FH and 0.01M EDTA on the activity of the classical (***Figure 5.14a***) and alternative (***Figure 5.14b***) pathways of complement activation in human serum was assessed using a commercial WeissLab ELISA. Reconstituted lyophilised human serum diluted 1 in 101 (***classical pathway***) or 1 in 18 (***alternative pathway)*** as the positive control, heat inactive human serum diluted 1 in 101; (***classical pathway***) or 1 in 18 (***alternative pathway***) as the negative control and human serum diluted 1 in 101 (***classical pathway***) or 1 in 18 (***alternative pathway***) alone or pre-incubated with either 250nM mouse anti-human FB, 250nM mouse anti-human MBL, 15nM mouse anti-human C1q blocking antibodies, 1.94µM purified human FH or 0.01M EDTA was incubated in duplicate on a plate either pre-coated with purified human IgM antibody (***classical pathway***) or LPS (***alternative pathway***). A buffer only control was also included in each assay.

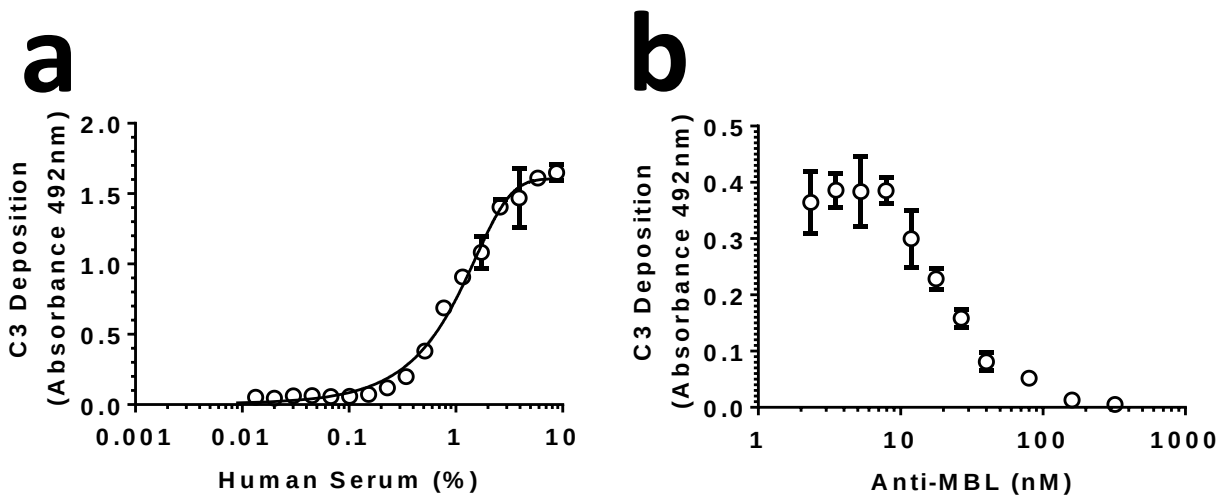


Figure 5. 13 Lectin Pathway Inhibition with an Anti-MBL Antibody

a, The activity of the classical, alternative and lectin pathways of human and rabbit complement towards *Neisseria meningitidis* serogroup W bacteria (strain 102/98) was assessed using three pathway-specific blocking antibodies. The concentration of the mouse anti-human MBL blocking antibody (R&D Systems) required to block the lectin pathway of complement activation in human serum was assessed by ELISA as described in ***Section 2.5.6***. ***a***, Firstly, the concentration of human serum resulting in 80% maximal C3 deposition (absorbance 492nm) was calculated. A range of serum concentrations (10-0.015%) was incubated on an ELISA plate coated with 200µg/mL mannan in duplicate. Subsequent C3 deposition at each concentration of human serum was detected with a sheep anti-human C3/C3c antibody. Each point represents the average absorbance achieved at each concentration of human serum. The error bars represent the standard deviation. The concentration of human serum resulting in 80% maximal C3 deposition was interpolated by fitting the data with a non-linear regression; sigmoidal, 4PL standard curve. The concentration of human serum required for 80% maximal C3 deposition was calculated as 2.0%. ***b***, Next, human (2.0%) serum was incubated with range of concentrations of the mouse anti-human MBL blocking antibody and added to an ELISA plate coated with 200µg/mL mannan in duplicate. Subsequent C3 deposition at each concentration of the mouse anti-human MBL blocking antibody was detected with a sheep anti-human C3/C3c antibody. Each point represents the average absorbance achieved at each concentration the mouse anti-human MBL blocking antibody. The error bars represent the standard deviation. The mouse anti-human MBL blocking antibody, at a concentration of 250nM, was sufficient to block lectin pathway of complement activation in human serum.

Subsequent C5b-9 deposition (absorbance 405nm) was detected with an anti-human C5b-9 antibody. The absorbance (405nm) achieved with human serum in the presence of each inhibitor was compared to the absorbance achieved with human serum alone by t-test statistical analysis. Compared to human serum alone, significantly reduced C5b-9 deposition was detected with the buffer only control, the negative control, 0.01M EDTA and 15nM of the mouse anti-human C1q blocking antibody (***Figure 5.14a***). Compared to human serum alone, significantly reduced C5b-9 deposition was detected with the buffer only control, the negative control, 0.01M EDTA, 250nM of the mouse anti-human FB blocking antibody and 1.94µM purified human FH (***Figure 5.14b***). It was concluded that the alternative pathway inhibitors (anti-FB and human FH) and lectin pathway inhibitor (anti-MBL) did not significantly impact on the classical pathway of human complement activation. In addition, it was concluded that the classical pathway inhibitor (anti-C1q) and lectin pathway inhibitor (anti-MBL) did not significantly impact on the alternative pathway of human complement activation.

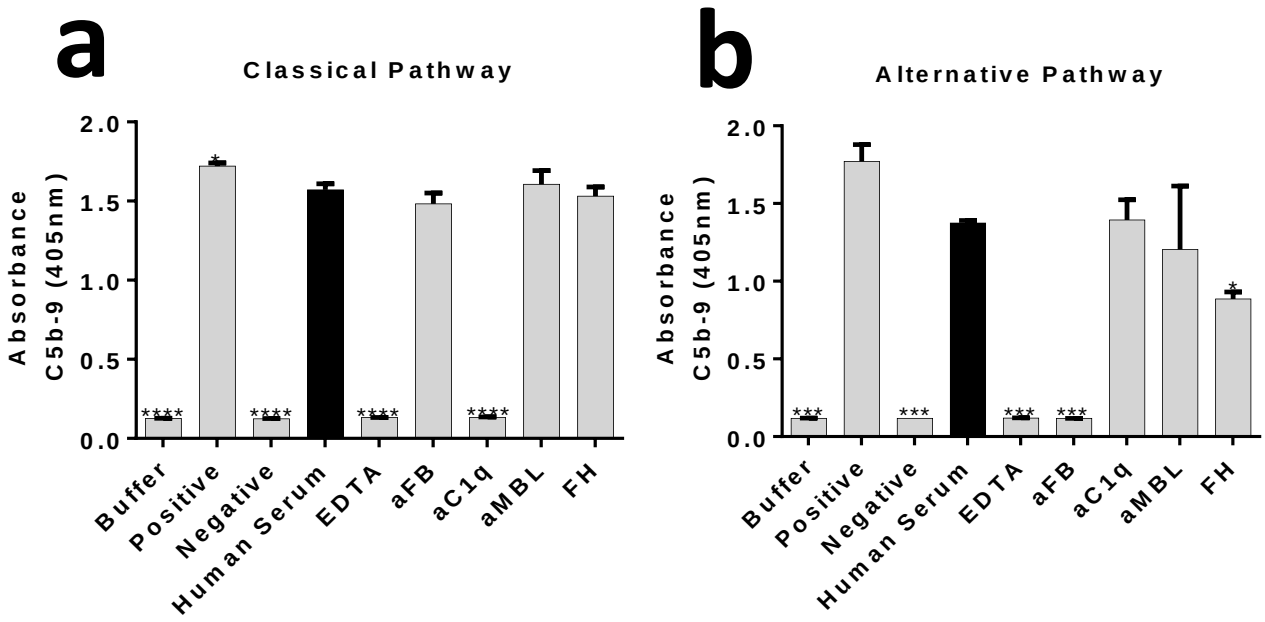


Figure 5. 14 Inhibition Classical and Alternative Pathway Activity – WeissLab Assay
The activity of the classical, alternative and lectin pathways of human and rabbit complement towards *Neisseria meningitidis* serogroup W bacteria (strain 102/98) was assessed using three pathway-specific blocking antibodies. The impact of 250nM mouse anti-human FB, 250nM mouse anti-human MBL and 15nM mouse anti-human C1q blocking antibodies as well as 1.94µM purified human FH and 0.01M EDTA on the activity of the classical (***a***) and alternative (***b***) pathways of complement activation in human serum was assessed using a commercial WeissLab ELISA. Reconstituted lyophilised human serum diluted 1 in 101 (***a***) or 1 in 18 (***b***) as the positive control, heat inactive human serum diluted 1 in 101 (***a***) or 1 in 18 (***b***) as the negative control and human serum (diluted 1 in 101 (***a***) or 1 in 18 (***b***); positive) alone or pre-incubated with either 250nM mouse anti-human FB, 250nM mouse anti-human MBL, 15nM mouse anti-human C1q blocking antibodies, 1.94µM purified human FH or 0.01M EDTA was incubated in duplicate on a plate either pre-coated with purified human IgM antibody (***a***) or LPS (***b***). A buffer only control was also included in each assay. The classical pathway assay buffer (***a***) consisted of veronal buffered saline (VBS) with 5mM $MgCl_2$, and the alternative pathway assay buffer consisted of VBS with 10mM EGTA and 5mM $MgCl_2$ (***b***). Subsequent C5b-9 deposition (absorbance 405nm) was detected with a anti-human C5b-9 antibody. Each bar represents the mean average absorbance achieved with each sample. The error bars represent the standard deviation. The absorbance (405nm) achieved with human serum in the presence of each inhibitor was compared to the absorbance achieved with human serum alone by t-test statistical analysis. The asterisks indicate the statistical significance of the difference between the average absorbance achieved with human serum alone and other conditions (*** = P value<0.001; **** = P value<0.0001; * = P value<0.05). ***a***, Compared to human serum alone, significantly reduced C5b-9 deposition was detected with the buffer only control, the negative control, 0.01M EDTA and 15nM of the mouse anti-human C1q blocking antibody. ***b***, Compared to human serum alone, significantly reduced C5b-9 deposition was detected with the buffer only control, the negative control, 0.01M EDTA, 250nM of the mouse anti-human FB blocking antibody and 1.94µM purified human FH.

5.3.2 Complement Deposition Assay

The activity of the classical, alternative and lectin pathways of human and rabbit complement towards *Neisseria meningitidis* serogroup W bacteria (strain 102/98) was assessed by flow cytometry using three pathway-specific blocking antibodies (***Figure 5. 15***). Killed *Neisseria meningitidis* serogroup W-135 (strain 102/98) bacteria was incubated in 5% IgG-depleted human serum (prepared as described by Brookes et al., 2013) or 5% rabbit serum in the absence (complement) or presence of 250nM mouse anti-human FB, 250nM mouse anti-human MBL and 15nM mouse anti-human C1q (human serum only) blocking antibodies as well as 1.94µM purified human FH. Bacteria were also incubated in buffer only as the negative control for the assay (bacteria only). Subsequent complement deposition (median fluorescence) was detected using a rabbit anti-human C3c FITC-conjugated antibody (Abcam). No source of serogroup W-135-specific antibody was introduced to the bacteria at any point during this assay.

The significant difference between complement deposition on the bacteria incubated with complement only and other preparations of bacteria were compared by t-test analysis. Compared to bacteria incubated with 5% IgG-depleted human serum alone significantly reduced complement deposition was detected in the presence of the mouse anti-human FB and mouse anti-human C1q blocking antibodies. (***Figure 5. 15a***). Anti-C1q and anti-FB antibody inhibited human complement activation by 98% and 31%, respectively. Addition of an anti-MBL antibody and human FH to IgG-depleted human serum had no significant impact on C3 deposition. Compared to bacteria incubated with 5% rabbit serum alone significantly reduced complement deposition was detected in the presence of mouse anti-human FB and mouse anti-human MBL blocking antibodies as well as purified human FH (***Figure 5. 15b***).

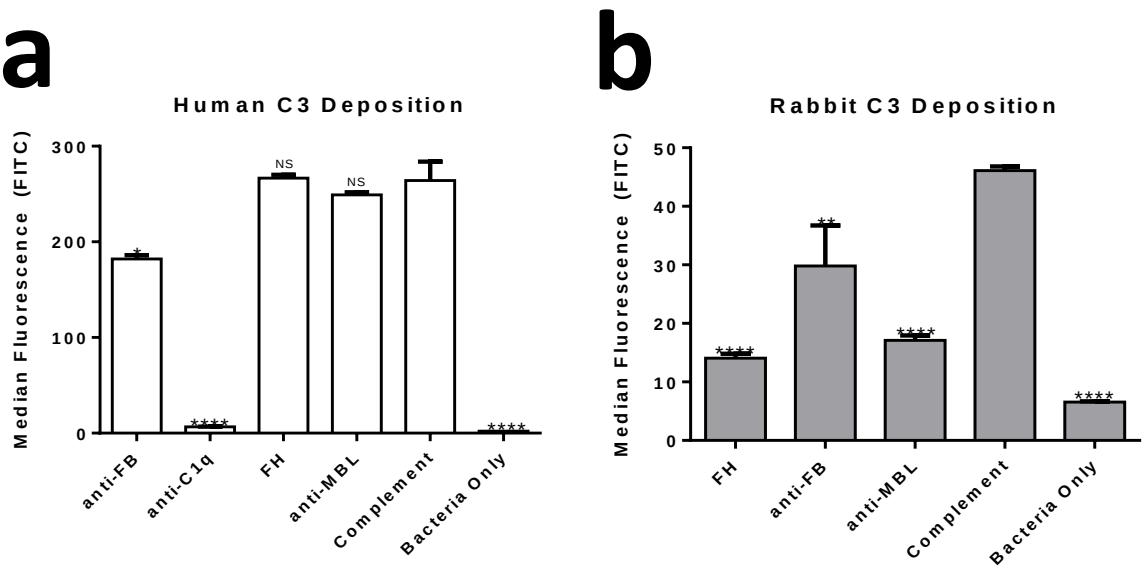


Figure 5. 15 Human and Rabbit C3 Deposition on *Neisseria meningitidis* Serogroup W-135 (strain 102/98) with Complement Pathway Inhibitors

The activity of the classical, alternative and lectin pathways of human and rabbit complement towards *Neisseria meningitidis* serogroup W bacteria (strain 102/98) was assessed using three pathway-specific blocking antibodies. ***a***, Killed *Neisseria meningitidis* serogroup W-135 (strain 102/98) bacteria was incubated in 5% IgG-depleted human serum (prepared as described by Brookes et al., 2013) in the absence (complement) or presence of 250nM mouse anti-human FB, 250nM mouse anti-human MBL and 15nM mouse anti-human C1q blocking antibodies as well as 1.94µM purified human FH. Bacteria were also incubated in buffer only as the negative control for the assay (bacteria only). Subsequent complement deposition (median fluorescence) was detected using a rabbit anti-human C3c FITC-conjugated antibody (Abcam). Each bar represents the average median fluorescence achieved with each preparation and the error bars represent the standard deviation. The significant difference between complement deposition on the bacteria incubated with complement only and other preparations of bacteria were compared by t-test analysis. The asterisks indicate the statistical significance of the difference between the average fluorescence achieved between complement deposition of bacteria incubated with complement only and the other preparations (**** = P value<0.0001; NS = not significant). Compared to bacteria incubated with 5% IgG-depleted human serum alone significantly reduced complement deposition was detected in the presence of the mouse anti-human FB and mouse anti-human C1q blocking antibodies. ***b***, Killed *Neisseria meningitidis* serogroup W-135 bacteria was incubated in 5% rabbit serum in the absence (complement) or presence of 250nM mouse anti-human FB and 250nM mouse anti-human MBL blocking antibodies as well as 1.94µM purified human FH. Bacteria were also incubated in buffer only as the negative control for the assay (bacteria only). Subsequent complement deposition (median fluorescence) was detected using a goat anti-rabbit C3c FITC-conjugated antibody (Acris). Each bar represents the average median fluorescence achieved with each preparation and the error bars represent the standard deviation. Compared to bacteria incubated with 5% rabbit serum alone significantly reduced complement deposition was detected in the presence of mouse anti-human FB and mouse anti-human MBL blocking antibodies as well as purified human FH. NB. No source of serogroup W-135-specific antibody was introduced to the bacteria at any point during this assay.

5.3.3 Opsonophagocytic Assay

The activity of the classical, alternative and lectin pathways of human and rabbit complement towards *Neisseria meningitidis* serogroup W bacteria (strain 102/98) was assessed by opsonophagocytic assay using three pathway-specific blocking antibodies (***Figure 5. 16***). Killed BCECF-stained *Neisseria meningitidis* serogroup W-135 (strain 102/98) bacteria were incubated in 5% IgG-depleted human serum (prepared as described by Brookes et al., 2013) or 5% rabbit serum in the absence (complement) or presence of 250nM mouse anti-human FB, 250nM mouse anti-human MBL and 15nM mouse anti-human C1q (human serum only) blocking antibodies as well as 1.94µM purified human FH. Bacteria were also incubated in buffer only as the negative control for the assay (bacteria only). Bacteria were then incubated with N,N-dimethylformamide differentiated HL60 cells (MOI=40). The amount of BCECF-AM-stained *Neisseria meningitidis* serogroup W-135 (strain 102/98) phagocytosed by differentiated HL60 cells was detected in the fluorescein channel. No source of serogroup W-135-specific antibody was introduced to the bacteria at any point during this assay.

The significant difference between phagocytosis of the bacteria incubated with complement only and other preparations of bacteria were compared by t-test analysis. Compared to bacteria incubated with 5% IgG-depleted human serum alone significantly reduced phagocytosis was detected in the presence of the 250nM mouse anti-human FB and 15nM mouse anti-human C1q blocking antibodies (***Figure 5. 16a***). Compared to bacteria incubated with 5% rabbit serum alone significantly reduced phagocytosis was detected in the presence of 250nM mouse anti-human FB and 250nM mouse anti-human MBL blocking antibodies as well as 1.94µM purified human FH (***Figure 5. 16b***).

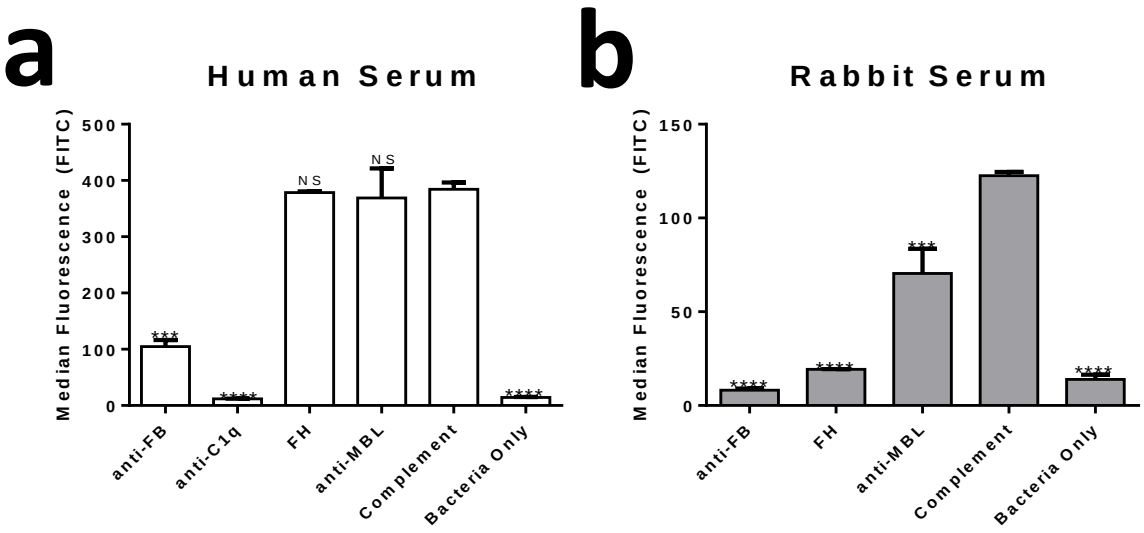


Figure 5. 16 Human and Rabbit Complement Opsonophagocytic Assay with *Neisseria Meningitidis* Serogroup W-135 (strain 102/98) and Complement Pathway Inhibitors

The activity of the classical, alternative and lectin pathways of human and rabbit complement towards *Neisseria meningitidis* serogroup W bacteria (strain 102/98) was assessed using three pathway-specific blocking antibodies. ***a***, Killed BCECF-stained *Neisseria meningitidis* serogroup W-135 (strain 102/98) bacteria were incubated in 5% IgG-depleted human serum (prepared as described by Brookes et al., 2013) in the absence (complement) or presence of 250nM mouse anti-human FB, 250nM mouse anti-human MBL and 15nM mouse anti-human C1q blocking antibodies as well as 1.94µM purified human FH. Bacteria were also incubated in buffer only as the negative control for the assay (bacteria only). Bacteria were then incubated with N,N-dimethylformamide differentiated HL60 cells. Subsequent phagocytosis (fluorescence) of the bacteria by the HL60 cells was assessed by flow cytometry. Each bar represents the average median fluorescence achieved with each preparation and the error bars represent the standard deviation. The significant difference between phagocytosis of the bacteria incubated with complement only and other preparations of bacteria were compared by t-test analysis. The asterisks indicate the statistical significance of the difference between the average fluorescence achieved between phagocytosis of bacteria incubated with complement only and the other preparations (** = P value<0.01; *** = P value<0.001; **** = P value<0.0001; NS = not significant). Compared to bacteria incubated with 5% IgG-depleted human serum alone significantly reduced phagocytosis was detected in the presence of the 250nM mouse anti-human FB and 15nM mouse anti-human C1q blocking antibodies. ***b***, Killed BCECF-stained *Neisseria meningitidis* serogroup W-135 (strain 102/98) bacteria was incubated in 5% rabbit serum in the absence (complement) or presence of 250nM mouse anti-human FB and 250nM mouse anti-human MBL blocking antibodies as well as 1.94µM purified human FH. Bacteria were also incubated in buffer only as the negative control for the assay (bacteria only). Bacteria were then incubated with N,N-dimethylformamide differentiated HL60 cells. Subsequent phagocytosis (fluorescence) of the bacteria by the HL60 cells was assessed by flow cytometry. Each bar represents the average median fluorescence achieved with each preparation and the error bars represent the standard deviation. The significant difference between phagocytosis of the bacteria incubated with complement only and other preparations of bacteria were compared by t-test analysis. Compared to bacteria incubated with 5% rabbit serum alone significantly reduced phagocytosis was detected in the presence of 250nM mouse anti-human FB and 250nM mouse anti-human MBL blocking antibodies as well as 1.94µM purified human FH. NB. No source of serogroup W-135-specific antibody was introduced to the bacteria at any point during this assay.

5.3.4 Serum Bactericidal Assay

The activity of the classical, alternative and lectin pathways of human and rabbit complement towards *Neisseria meningitidis* serogroup W bacteria (strain 102/98) was assessed by SBA using three pathway-specific blocking antibodies (***Figure 5. 17***). Live *Neisseria meningitidis* serogroup W-135 (strain 102/98) bacteria was incubated in 25% IgG-depleted human serum in the absence (C') or presence of 250nM mouse anti-human FB, 250nM mouse anti-human MBL and 15nM mouse anti-human C1q blocking antibodies as well as 1.94µM purified human FH. Bacteria were also incubated in 25% heat-inactivated IgG-depleted human serum only as the negative control for the assay (HI C'). Each preparation of bacteria was grown on a blood agar plate overnight and the number of colony forming units (CFU) was counted for each. The significant difference between the number of CFU for bacteria incubated with serum only and the other preparations was assessed by t-test statistical analysis.

Compared to bacteria incubated with 25% IgG-depleted human serum only, a significantly higher number of CFUs were measured in the presence of 15nM mouse anti-human C1q blocking antibody or heat-inactivated serum. No source of serogroup W-135-specific antibody was introduced to the bacteria at any point during this assay (***Figure 5. 17a***). Compared to bacteria incubated with 25% rabbit serum only, a significantly higher number of CFUs were measured in the presence of 250nM mouse anti-human FB blocking antibody, heat-inactivated serum and 1.94 human FH (***Figure 5. 17b***).

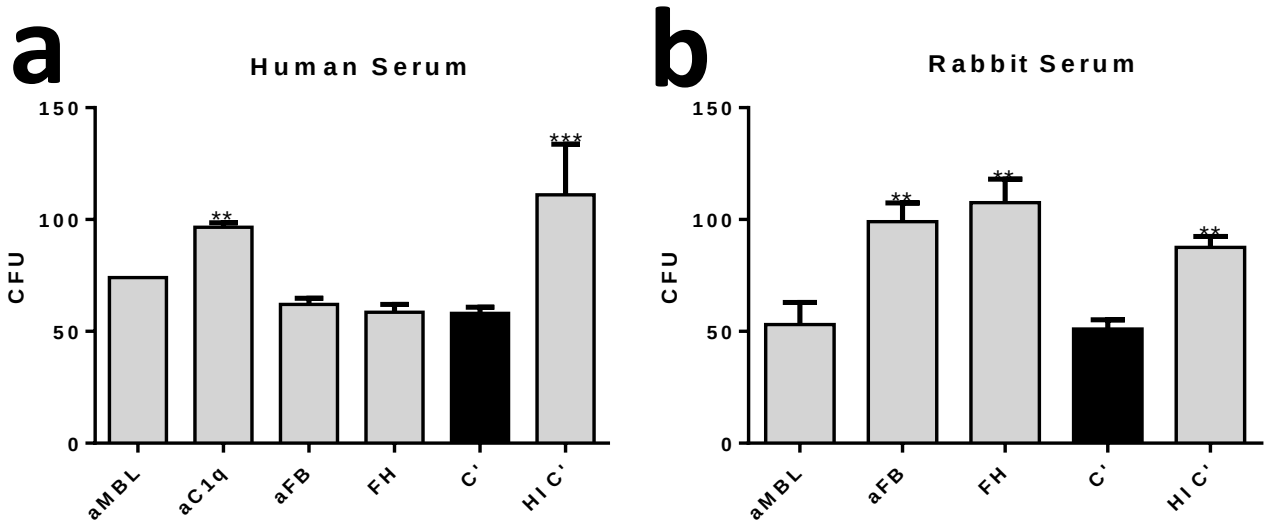


Figure 5. 17 Growth of *Neisseria meningitidis* Serogroup W-135 (strain 102/98) with Human and Rabbit Serum in the Presence of Complement Pathway Inhibitors

The activity of the classical, alternative and lectin pathways of human and rabbit complement towards *Neisseria meningitidis* serogroup W bacteria (strain 102/98) was assessed using three pathway-specific blocking antibodies. ***a***, Live *Neisseria meningitidis* serogroup W-135 (strain 102/98) bacteria was incubated in 25% IgG-depleted human serum in the absence (C') or presence of 250nM mouse anti-human FB, 250nM mouse anti-human MBL and 15nM mouse anti-human C1q blocking antibodies as well as 1.94µM purified human FH. Bacteria were also incubated in 25% heat-inactivated IgG-depleted human serum only as the negative control for the assay (HI C'). Each preparation of bacteria was grown on a blood agar plate overnight and the number of colony forming units (CFU) was counted for each. Each bar represents the average CFU achieved with each preparation and the error bars represent the standard deviation. The significant difference between the number of CFU for bacteria incubated with serum only and the other preparations was assessed by t-test statistical analysis. The asterisks indicate the statistical significance of the difference between the average CFU between complement deposition of bacteria incubated with serum only and the other preparations (** = P value<0.01; *** = P value<0.001). Compared to bacteria incubated with 25% IgG-depleted human serum only, a significantly higher number of CFUs were measured in the presence of 15nM mouse anti-human C1q blocking antibody or heat-inactivated serum. ***b***, Live *Neisseria meningitidis* serogroup W-135 (strain 102/98) bacteria was incubated in 25% rabbit serum in the absence (C') or presence of 250nM mouse anti-human FB and 250nM mouse anti-human MBL blocking antibodies as well as 1.94µM purified human FH. Bacteria were also incubated in 25% rabbit serum only as the negative control for the assay (HI C'). Each preparation of bacteria was grown on a blood agar plate overnight and the number of colony forming units (CFU) was counted for each. Each bar represents the average CFU achieved with each preparation and the error bars represent the standard deviation. The significant difference between the number of CFU for bacteria incubated with serum only and the other preparations was assessed by t-test statistical analysis. Compared to bacteria incubated with 25% rabbit serum only, a significantly higher number of CFUs were measured in the presence of 250nM mouse anti-human FB blocking antibody, heat-inactivated serum and 1.94 human FH. NB. No source of serogroup W-135-specific antibody was introduced to the bacteria at any point during this assay.

The impact of 1.94µM purified human FH on the growth of Neisseria meningitidis serogroup W-135 (strain 102/98) in the presence of either 25% IgG-depleted human serum or 25% rabbit serum was assessed by serum bactericidal assay (***Figure 5.19***). Live *Neisseria meningitidis* serogroup W-135 (strain 102/98) bacteria were incubated with heat-inactivated mouse anti-meningococcal polysaccharide serum (diluted 1 in 2, 1 in 4 or 1 in 8) or buffer only (HI t=0). The bacteria were then incubated with either 25% IgG-depleted human serum only or 25% IgG-depleted human serum supplemented with 1.94µM purified human FH. Each preparation of bacteria was then grown on a blood agar plate overnight and the number of colony forming units (CFU) was counted for each. By t-test analysis, there no significant difference between the number of CFUs incubated with either 25% IgG-depleted human serum only or 25% IgG-depleted human serum supplemented with 1.94µM purified human FH at any dilution of the heat-inactivated mouse anti-meningococcal polysaccharide serum (***Figure 5.19a***). By t-test analysis, there was a significant increase in the number of CFUs with 25% rabbit serum supplemented with 1.94µM purified human FH compared to 25% rabbit serum only with the heat-inactivated mouse anti-meningococcal polysaccharide serum diluted 1 in 2 and 1 in 4 (* = P value<0.05; ***Figure 5.19b***).

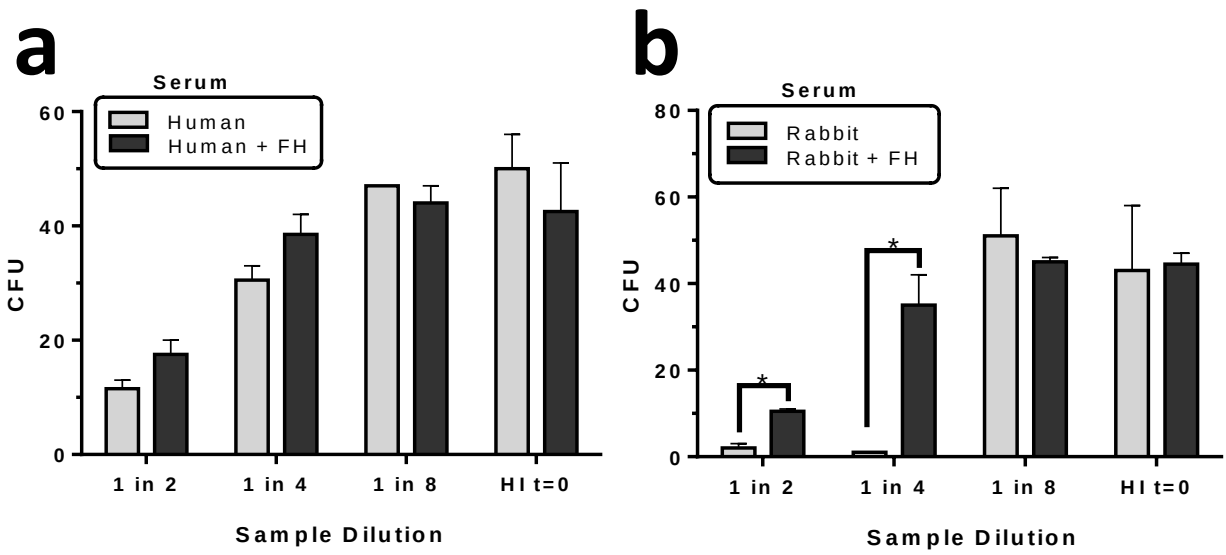


Figure 5. 18 Growth of *Neisseria meningitidis* Serogroup W-135 (strain 102/98) with Human or Rabbit Serum and Human FH

The impact of 1.94µM purified human FH on the growth of Neisseria meningitidis serogroup W-135 (strain 102/98) in the presence of either 25% IgG-depleted human serum (**a**) or 25% rabbit serum (**b**) was assessed by serum bactericidal assay. **a**, Neisseria meningitidis serogroup W-135 (strain 102/98) bacteria were incubated with heat-inactivated mouse anti-meningococcal polysaccharide serum (diluted 1 in 2, 1 in 4 or 1 in 8) or buffer only (HI t=0). The bacteria were then incubated with either 25% IgG-depleted human serum only (grey bars) or 25% IgG-depleted human serum supplemented with 1.94µM purified human FH (black bars). Each preparation of bacteria was then grown on a blood agar plate overnight and the number of colony forming units (CFU) was counted for each. Each bar represents the average CFU achieved with each preparation and the error bars represent the standard deviation. By t-test analysis, there no significant difference between the number of CFUs incubated with either 25% IgG-depleted human serum only or 25% IgG-depleted human serum supplemented with 1.94µM purified human FH at any dilution of the heat-inactivated mouse anti-meningococcal polysaccharide serum. **b**, Neisseria meningitidis serogroup W-135 (strain 102/98) bacteria were incubated with heat-inactivated mouse anti-meningococcal polysaccharide serum (diluted 1 in 2, 1 in 4 or 1 in 8) or buffer only (HI t=0). The bacteria were then incubated with either 25% rabbit serum only (grey bars) or 25% rabbit serum supplemented with 1.94µM purified human FH (black bars). Each preparation of bacteria was then grown on a blood agar plate overnight and the number of colony forming units (CFU) was counted for each. Each bar represents the average CFU achieved with each preparation and the error bars represent the standard deviation. By t-test analysis, there was a significant increase in the number of CFUs with 25% rabbit serum supplemented with 1.94µM purified human FH compared to 25% rabbit serum only with the heat-inactivated mouse anti-meningococcal polysaccharide serum diluted 1 in 2 and 1 in 4 (* = P value<0.05).

5.4 Discussion

5.4.1 Interaction of Antibody Subclasses with Human and Rabbit Complement

The first aim of this study was to compare the ability of human IgM and IgG subclasses to activate human and rabbit complement. Firstly, complement activation after incubation of either human or rabbit serum with different solid-phase antibody subclasses was measured by ELISA. C3 deposition was detected as a marker of complement activation using an anti-human/rabbit C3 HRP-conjugated antibody. The anti-C3 antibody was determined to be cross-reactive to both human and rabbit C3 by western blot (***Figure 5. 1a***). Purified antibody subclasses were immobilised in two ways: either directly coated onto ELISA plates or by binding antigen-specific antibodies to ELISA plates coated directly with meningococcal polysaccharides from serogroups A, C, W and Y.

Human IgG1, IgG2, IgG3, IgG4, IgM, anti-MenACWY IgG/IgM, human IgG and rabbit IgG antibody preparations were directly coated to ELISA plates, incubated with either human or rabbit serum and subsequent complement activation (C3 deposition) was detected (***Figure 5. 4***). By rank order of complement activation IgG3 antibody activated human complement to the greatest extent followed by IgG1 > IgM > IgG2 > IgG4. By rank order of complement activation IgG3 antibody activated rabbit complement to the greatest extent followed by IgM > IgG1 > IgG2 > IgG4. The differential ability of these antibody preparations to activate human and rabbit complement correlate with their ability to bind human and rabbit C1q as shown in ***Chapter 4: Figure 4.9*** and ***Chapter 4: Figure 4.17***. As discussed in ***Chapter 4: Section 4.4.2***, the differential ability of IgM and human IgG subclasses to activate human complement are well known and mirror these data (Bindon et al., 1988; Brüggemann et al., 1987; Kaul and Loos, 1997; Schumaker et al., 1976). I have extended these observations to include comparison with rabbit serum and

highlighted significant differences with the ability of human antibody subclasses to activate human complement.

In vivo, the classical pathway of complement is activated upon the binding of C1q to antigen-bound immunoglobulins (Bindon et al., 1988; Brüggemann et al., 1987; Kaul and Loos, 1997; Schumaker et al., 1976). In the case of IgM, binding to antigen reveals the C1q binding site on the CH3 and CH4 domains of IgM allowing C1q to associate, activating complement (Czajkowsky and Shao, 2009; Feinstein and Munn, 1969; Perkins et al., 1991; Zlatarova et al., 2006). In the case of IgG, binding to antigen arranges the Fc region in such a way to facilitate the formation of hexamers that generate an efficient dock for C1q to bind at a high affinity, activating complement (Diebolder et al., 2014). As such, directly coating antibody to ELISA plates could be considered an artificial surface and not conducive to activating complement in the physiological manner. To address this, an assay was designed to capture antigen-specific antibody to ELISA plates coated with antigen; thereby simulating the mechanism of complement activation in vivo. Anti-MenACWY IgG1, IgG2 and IgM antibodies were purified human plasma taken from 14 individuals (50mL/person) vaccinated with meningococcal polysaccharides from serogroups A, C, W and Y. Antibody preparations were assessed for purity by SDS-PAGE (***Figure 5. 5***). By coomassie stain, each antibody preparation (reduced) appeared as two bands corresponding to the heavy and light chains of antibody (***Figure 5. 5a***). No contamination of each antibody preparation with other antibody subclasses was detected by western blot (***Figure 5. 5b***). Each antibody preparation was bound to ELISA plates coated with meningococcal polysaccharides from serogroups A, C, W and Y, incubated with either human or rabbit serum and subsequent complement activation was detected and compared between species. Anti-MenACWY IgG3 and IgG4 antibodies were not isolated at sufficient quantities for this assay. By order of complement activation: human IgG1 activated human complement more than IgM

followed by IgG2 (***Figure 5. 7a***). In contrast, human IgM antibody activate rabbit serum more than IgG1 followed by IgG2 (***Figure 5. 7b***).

These data confirm the differences in the abilities of human antibody subclasses to activate human and rabbit complement previously suggested by other studies and further define the mechanisms responsible for differential bactericidal activities of human and rabbit complement against *Neisseria meningitidis* (Griffiss and Goroff, 1983; Mandrell et al., 1995; Santos et al., 2001; Zollinger and Mandrell, 1983). The data from this study have confirmed that human IgM is a more potent subclass in its ability to activate rabbit complement than it is to activate human complement and human IgG1 is a more potent subclass in its ability to activate human complement than it is to activate rabbit complement. Again, these data further question the use of rabbit serum as the source of complement in SBAs and provide significant insight into difficulties and challenges associated with interpretation of rSBA data.

5.4.1.1 Complement Activation by Anti-MenACWY Antibody Subclasses Bound to Neisseria meningitidis

Complement activation by purified anti-MenACWY IgG1, IgG2 and IgM antibodies incubated with *Neisseria meningitidis* serogroup W-135 (strain 102/98) bacterium in the presence of human serum was investigated by SBA and complement deposition assay (***Figure 5. 9***). Despite binding to the bacterium, no significant C3b deposition nor inhibition of bacterial growth could be detected with each of the purified anti-MenACWY antibody subclasses. As such, the differential ability of these human antibody subclasses to activate human and rabbit complement, when bound to *Neisseria meningitidis* serogroup W-135 (strain 102/98), could not be compared.

5.4.2 Human and Rabbit Complement Pathways and Neisseria Meningitidis

The second aim of this project was to assess the contribution of the different complement cascade pathways to bactericidal killing with rabbit or human complement. The impact of blocking each pathway of complement activation in human and rabbit serum on complement activation, subsequent opsonisation and phagocytosis and survival of *Neisseria meningitidis* serogroup W-135 (strain 102/98) was assessed. Furthermore, the consequences of adding purified human FH to both human and rabbit serum on complement activation with *Neisseria meningitidis* serogroup W-135 (strain 102/98) was also assessed. These assays were performed with *Neisseria meningitidis* serogroup W-135 (strain 102/98) only and further work comparing the interaction of each pathway of human and rabbit complement activation with other important serogroups (A, B, C and Y) would be of interest.

The ability of an anti-C1q antibody to block classical pathway activation in human and rabbit serum was assessed using a classical pathway haemolytic assay and WeissLab complement screen assay (***Figure 5. 11*** and ***Figure 5. 14a***). Human complement activation by the classical pathway was effectively blocked by the anti-C1q antibody. However, the anti-C1q antibody was not cross-reactive with rabbit C1q and did not block rabbit complement activation by the classical pathway. Specificity of the anti-C1q blocking antibody to human C1q and not to rabbit C1q was confirmed by western blot (***Figure 5. 10b***). Classical pathway inhibition of human complement significantly reduced complement deposition on, and subsequent phagocytosis of, *Neisseria meningitidis* serogroup W-135 (strain 102/98) as well as increasing survival in human serum (***Figure 5. 15a***, ***Figure 5. 16a*** and ***Figure 5. 17a***). Direct C1q binding and classical pathway activation on Gram-negative bacteria has been described although, its importance in the absence of bactericidal antibody is probably minimal (Agarwal et al., 2014; Drogari-Apiranthitou et al., 2002; Mintz et al., 1995; Tenner et al., 1984). However, these data show that the classical pathway of human complement is responsible for the majority of complement activation on

Neisseria meningitidis. Further work investigating the contribution of rabbit classical pathway of complement activation with *Neisseria meningitidis* is required.

The ability of an anti-FB antibody to block alternative pathway activation in human serum was assessed using an alternative pathway haemolytic assay and WeissLab complement screen assay (***Figure 5. 12*** and ***Figure 5. 14b***). The cross-reactivity of a known blocking anti-FB antibody with human and rabbit FB was confirmed by western blot (***Figure 5. 10a***). The ability of the anti-FB antibody to block alternative pathway activation in rabbit serum was not assessed here but has been shown in a previous study (Subías et al., 2014). Alternative pathway inhibition had a significantly greater impact on complement activation and subsequent phagocytosis of *Neisseria meningitidis* serogroup W-135 (strain 102/98) in presence of rabbit serum than with human serum (***Figure 5. 15b*** and ***Figure 5. 16b***). Whereas alternative pathway inhibition significantly increases the growth of *Neisseria meningitis* in rabbit serum, no impact on bacterial growth was seen when blocking the alternative pathway of human serum (***Figure 5. 17***). These data suggest that the alternative pathway of rabbit complement contributes more to complement activation towards *Neisseria meningitidis* serogroup W-135 (strain 102/98) than the alternative pathway of human complement. The FHbp, expressed by *Neisseria meningitidis*, is able to effectively downregulate the alternative pathway of complement activation by binding FH (Borrow et al., 2001a; Jodar et al., 2000; Maslanka et al., 1997; Zollinger and Mandrell, 1983). FHbp is specific for human FH and cannot bind rabbit FH (Granoff et al., 2009). As such, *Neisseria meningitidis* cannot regulate the alternative pathway of rabbit complement. These data confirm that the alternative pathway of human complement is effectively blocked by the bacterium thus explaining why further inhibition had only a small impact on complement activation compared to alternative pathway inhibition of rabbit complement.

The ability of an anti-MBL antibody to block lectin pathway activation in human serum was assessed by functional ELISA (***Figure 5. 13b***). The cross-reactivity of the anti-MBL antibody with human and rabbit MBL was confirmed by western blot (***Figure 5. 10c***). The ability of the anti-MBL antibody to block lectin pathway activation in rabbit serum was not assessed and further work characterising this function of this antibody is required. Lectin pathway inhibition reduced complement activation and subsequent phagocytosis of *Neisseria meningitidis* serogroup W-135 (strain 102/98) significantly more in the presence of rabbit serum than with human serum (***Figure 5. 15b*** and ***Figure 5. 16b***). These data suggest that the lectin pathway of rabbit complement contributes more to complement activation towards *Neisseria meningitidis* serogroup W-135 (strain 102/98) than the lectin pathway of human complement. Despite reducing rabbit C3 deposition on bacterium by roughly 40%, lectin pathway inhibition did not impact on bacterial growth (***Figure 5. 17b***). MBL, the initiator of the lectin pathway of complement, has been shown to directly bind to *Neisseria meningitidis* following incubation with human serum (Estabrook et al., 2004; Sprong et al., 2004). It is unclear whether complement activation by the lectin pathway plays a significant role in the clearance of *Neisseria meningitidis*. One study showed no difference between complement deposition of *Neisseria meningitidis* following incubation with serum taken from either MBL sufficient or deficient individuals suggesting a minimal role of the lectin pathway (Hellerud et al., 2010). Other studies have shown significantly reduced complement activation when the lectin pathway was inhibited or absent (Jack et al., 2001; Sprong et al., 2003). The data from this study show little impact on complement activation when the lectin pathway in inhibited agreeing a limited role for the lectin pathway in the clearance of *Neisseria meningitidis*. It must be noted that MBL deficient children do show an increased risk to meningococcal disease highlighting the importance of either the ability of MBL to activate complement or promote phagocytosis of *Neisseria meningitidis* in early

life (Hibberd et al., 1999; Jack et al., 1998; Jack et al., 2001; van Helden and Hoal-van Helden, 1999).

Addition of purified human FH to rabbit serum significantly decreased the bactericidal activity of HI immune serum towards *Neisseria meningitidis* serogroup W-135 (strain 102/98) to levels comparable to human serum (with or without additional human FH) (***Figure 5. 18***). In the absence of bactericidal antibody, the addition of FH to rabbit serum increased the growth of *Neisseria meningitidis* serogroup W-135 (strain 102/98) as well as decreasing complement deposition and phagocytosis of the bacterium (***Figure 5. 15b***, ***Figure 5. 16b*** and ***Figure 5. 17b***). A study has shown that human FH, bound to *Neisseria meningitidis*, is able to regulate both human and rabbit complement activity (Granoff et al., 2009). The data from this study confirm that human FH can regulate rabbit complement activity towards *Neisseria meningitidis* in SBAs normalising SBA titres to similar levels seen with human serum. In the absence or presence of bactericidal antibody, addition of FH to human serum did not significantly impact on the growth of *Neisseria meningitidis*, complement deposition on nor phagocytosis of the bacterium (***Figure 5. 15a***, ***Figure 5. 16a*** and ***Figure 5. 17a***). These data suggest the level of FH present in HI immune serum and human complement is sufficient to fully saturate the FHbp expressed by the bacteria in each assay.

It is concluded that, in the absence of bactericidal antibody, human complement activation is primarily driven by the classical activation with minimum input from the alternative and lectin pathways. Furthermore, the alternative pathway of rabbit complement plays a much more significant role in complement activation towards *Neisseria meningitidis* serogroup W-135 (strain 102/98) than the alternative pathway of human complement. However, it cannot be concluded whether rabbit complement activation on *Neisseria meningitidis* is initiated by the classical, alternative or lectin pathway. Further work is needed to distinguish between the contributions of each pathway in rabbit complement.

Chapter Six – Discussion

6.1 Interaction of Human and Rabbit Complement with Antibody Subclasses

The SBA measures bactericidal activity of sera towards *Neisseria meningitis* and is used to assess the efficacy of new meningococcal vaccines for licensure (Goldschneider et al., 1969a; WHO, 1976, 2006). It is generally considered that individuals with an SBA titre greater than four are protected against invasive meningococcal disease (Goldschneider et al., 1969a; Goldschneider et al., 1969b; Gotschlich et al., 1969a).

The standard protocol for the SBA uses BRS as the source of complement due to the high frequency of endogenous bactericidal activity present in human serum (Maslanka et al., 1997). The use of BRS in SBAs is controversial as SBA titres achieved with rabbit serum are significantly higher than with human serum (Borrow et al., 2001a; Jodar et al., 2000; Maslanka et al., 1997; Zollinger and Mandrell, 1983). As such, individuals lacking bactericidal activity (SBA titre≤4) when assessed by hSBAs often achieve bactericidal titres greater than 4 when assessed by rSBA resulting in a high rate of false positives. A further concern is that rSBA titres correlate poorly with hSBA titres (Brookes et al., 2013; Gill et al., 2011a; Santos et al., 2001; Zollinger and Mandrell, 1983).

One reason that rSBA titres are significantly higher than hSBA titres may be due to species-specific differences between the interaction of *Neisseria meningitis* and complement regulators. However, these species-specific differences in the ability of *Neisseria meningitis* to regulate human and rabbit complement are constant and do not account for the poor correlation between the two assays. I hypothesised that the differential ability of human antibody subclasses to activate human and rabbit complement is responsible for the lack of

specificity for the rSBA in predicting hSBA titres. The main focus of this project was to investigate the differences between the interaction of human antibody subclasses with human and rabbit complement and the implications for the use of rabbit serum as a source of complement in SBAs.

Firstly, the relationship between the concentration of *Neisseria meningitidis* serogroup A, C W and Y polysaccharide-specific IgG1, IgG2 and IgM antibody present in immune plasma with hSBA and rSBA titres was assessed (***Chapter 3: Table 3.4*** and ***Table 3.6***). Serum antibody concentrations were measured either one or four months post vaccination with a plain (Mencevax™) or TT-conjugated (Nimenrix™) quadrivalent polysaccharide vaccine. In infants and children, the primary antibody subclass response to both vaccines was IgM and IgG1 with minimal levels of IgG2 (***Chapter 3: Table 3.3*** and ***Table 3.5***). This differed significantly to the polysaccharide-specific antibody subclass composition in a cohort of adults where the primary antibody subclass response to vaccination was in the order IgG2, IgG1 and IgM (***Chapter 3: Figure 3.1***). In cohorts receiving the TT-conjugated quadrivalent polysaccharide vaccine, hSBA titres correlated best with the serum concentration of polysaccharide-specific IgG1 antibody whereas rSBA correlated best with the serum concentration of polysaccharide-specific IgM antibody. To further define these species-specific differences, the interaction of the human antibody subclasses IgG1, IgG2, IgG3, IgG4 and IgM with human and rabbit complement was investigated at the level of C1q and activated fragments of C3 (***Chapter 4: Figure 4.9*** and ***Chapter 5: Figure 5.4***). In both assays, the rank of order of antibody subclasses to fix human complement was IgG3>IgG1>IgM>IgG2>IgG4 whereas the rank order of antibody subclasses to fix rabbit complement was IgG3>IgM>IgG1>IgG2>IgG4. The affinity of purified human and rabbit C1q to human IgG1, IgG2, IgG3 and IgG4 antibody was assessed by SPR (***Chapter 4: Table 4.1***). The rank order of both human and rabbit C1q affinity to human IgG subclasses was IgG3>IgG1>IgG2>IgG4. In general, rabbit C1q displayed lower affinities for the four subclasses compared to human C1q. The greatest difference in KD between human and rabbit C1q was seen with IgG3 (ratio of 1:3.3)

followed by IgG1 (1:1.4), IgG2 (1:1.2) and IgG4 (1:1.1). Although the affinity of human and rabbit C1q to human IgM antibody could not be established in this project, we hypothesise a higher affinity of rabbit C1q. Finally, the ability of purified human polysaccharide-specific IgG1, IgG2 and IgM antibody to fix human and rabbit complement when bound to meningococcal polysaccharides was investigated by ELISA (***Chapter 5: Figure 5.7***). As before, the rank order of subclasses to fix human complement was IgG1>IgM>IgG2 whereas the rank order of antibody subclasses to fix rabbit complement was IgM>IgG1>IgG2.

These data define important differences in the ability of human antibody subclasses to fix human and rabbit complement. Specifically, it appears that polysaccharide-specific IgM antibody contributes significantly more to bactericidal titres in SBAs using BRS as the source of complement and that polysaccharide-specific IgG1 antibody contributes significantly more to bactericidal titres in SBAs using human serum as the source of complement. As such, it is likely that individuals with higher concentrations of serogroup-specific IgM antibody in response to meningococcal vaccination positively skews rSBA titres compared to hSBA titres. These data also suggest that polysaccharide-specific IgG3 antibody contributes significantly more to bactericidal titres in SBAs using human serum as the source of complement. However, the IgG3 response to TT-conjugated quadrivalent polysaccharide vaccine is minimal and unlikely to greatly impact on differences between hSBA and rSBA titres (***Chapter3: Figure 3.1***).

SBA titres are usually assessed one month post vaccination during the early stages of immune response to vaccination. As higher anti-polysaccharide IgM titres result in disproportionally higher rSBA titres compared to hSBA titres, it may be concluded that rSBA titres measured at one month post vaccination are misleadingly high and further question the relevance of BRS as the source of complement in SBAs in the licensure of new meningococcal vaccines.

6.1.1 Antibody Response to Plain versus TT-Conjugated Quadrivalent Meningococcal Polysaccharide Vaccines

In contrast to the antibody response to the TT-conjugated quadrivalent meningococcal polysaccharide vaccine, the antibody responses to the plain quadrivalent meningococcal polysaccharide vaccine correlated poorly with SBA titres (***Chapter3: Table 3.3***).

Higher affinity antibody responses to meningococcal vaccines are associated with higher SBA titres (Hetherington and Lepow, 1992; Schlesinger et al., 1992). In this way, it is thought that lower affinity antibodies contribute little to SBA titres. Furthermore, it is likely that the standard ELISA developed to measure the concentration of serogroup-specific antibody detects antibodies of both high and low affinity (Granoff et al., 1998). If this is this case, the inclusion of low-affinity antibody in the quantitation of serogroup-specific antibody may explain the poor correlation between antibody concentration and SBA titres following vaccination with the plain quadrivalent meningococcal polysaccharide vaccine.

These data also suggest that the significantly higher SBA titres achieved with the TT-quadrivalent meningococcal polysaccharide vaccine compared to the plain vaccine may not only be explained by the increase in concentration of antibody, but also by increased affinity maturation of the antibody secreted; a characteristic of TD antigens. Correlations may be improved with a modification to the ELISA that can distinguish between high and low-affinity antibodies. As shown in a previous study, this may be achieved by diluting samples in a buffer containing ammonium thiocyanate (Granoff et al., 1998). This will allow only antibodies only of the highest affinity to bind to the meningococcal polysaccharide-coated ELISA plates.

6.1.2 Anti-Meningococcal Polysaccharide Complement Deposition Assay

A functional assay was set up to measure the ability of purified vaccine-specific antibody to activate complement when bound to antigen (***Chapter 5: Figure 5.7***). However, this assay could easily be modified to measure the ability of vaccine-specific antibody present in vaccinee sera to activate complement when bound to antigen. In this way, the Meningococcal Complement Deposition ELISA may be used as simple assay to measure the efficacy of vaccines thus avoiding the numerous variables and issues present in the SBAs. For example, the Meningococcal Complement Deposition ELISA overcomes the issue of finding a suitable source of human complement lacking endogenous activity towards *Neisseria meningitidis*. This is achieved by depletion of vaccine antigen-specific antibody by affinity chromatography which can be completed on a large scale with multiple human donors (***Chapter 5: Figure 5.6***). For this assay to be used as a reliable correlate of protection, several steps of validation must be performed including calculating a suitable threshold for complement activation and dilution factor of vaccinee sera for an individual to be considered protected against disease. It may be necessary to include a positive control, known to contain a protective level of complement activating vaccine antigen-specific antibody, in each assay for comparison.

6.1.3 Bactericidal Activity of Purified Polysaccharide-Specific Antibodies

The bactericidal activity of purified polysaccharide-specific human IgG1, IgG2 and IgM antibody was investigated by SBA and complement deposition assay (***Chapter 5: Figure 5.9***). To our surprise these antibodies did not fix complement to *Neisseria meningitidis*. The mechanism behind the inability of these purified polysaccharide-specific antibody subclasses to activate complement, once bound to the bacteria, is unknown.

IgG-depleted human serum was used as the source of complement in these assays, prepared as described by Brookes et al., (2013). Briefly, IgG was removed by injection of human

serum through a Protein G Sepharose column. In addition to removing IgG, the serum was also depleted of C1q and C5 which were eluted from the Protein G column using a high salt buffer and added back to the IgG-depleted serum. The final concentration of C5 and C1q in the IgG-depleted serum was comparable to the pre-depleted serum. The concentration of FH, properdin and C3 in the IgG-depleted serum was assessed and were unaffected. The concentrations of other components were not measured. One reason for the negative killing data may be that other components are also depleted during the process of IgG depletion that are necessary for full complement activation with purified polysaccharide-specific antibody. In contrast to the purified anti-polysaccharide antibodies, immune serum significantly activated complement on the surface of *Neisseria meningitidis*. Immune serum may be replenishing this 'component' that is removed during the process of IgG depletion, which is not present in purified antibody.

Personal communications from Dr Stephen Taylor (Public Health England, Porton Down, Salisbury, UK), revealed that this phenomenon has been noted previously and it appears that the inability of purified antibody to activate complement when bound to *Neisseria meningitidis* is consistent only with antibody specific to polysaccharide. In contrast, antibodies specific to proteins expressed by *Neisseria meningitidis* activate complement significantly better than polysaccharide-specific antibodies when bound to the bacteria in equal concentrations (data not published). The mechanisms responsible for the differential ability of purified anti-polysaccharide and anti-protein antibody to activate complement on *Neisseria meningitidis* have not yet been investigated. It is possible that polysaccharide-specific antibody activates complement relatively more superficially compared to protein-specific antibody resulting in reduced bactericidal activity. Similar antigen-specific differences in the bactericidal activity of antibody have been previously described for the Gram-negative bacteria *Escherichia coli* (Howard and Glynn, 1971).

Meningococcal polysaccharide conjugate vaccines are effective and have reduced the burden of disease in countries where they have been successfully introduced. In the UK, routine meningococcal polysaccharide serogroup C conjugate vaccination was introduced in 1999 and the number of confirmed cases of meningococcal disease caused by serogroup C has decreased from 883 in 1999/2000 to just 28 in 2014/2015 (Gov.uk, 2015). Furthermore, I have shown that the concentration of polysaccharide-specific IgG1 and IgM antibodies present in immune serum correlates well with bactericidal titres (***Chapter 3: Table3.4*** and ***Table 3.6***). Therefore, polysaccharide-specific antibodies must be bactericidal and convey protection against invasive meningococcal disease. It appears that polysaccharide-specific antibodies are not bactericidal alone but do contribute to bactericidal titres when present in immune serum (***Chapter 5: Figure 5.9***) (Elias et al., 2013; Lieberman et al., 1996; Maslanka et al., 1998). In contrast, protein-specific antibodies are bactericidal alone and when present in immune serum. As such, it may be concluded that the missing 'component' removed from the IgG-depleted complement that is required for polysaccharide-specific antibodies to be bactericidal is protein-specific antibody (***Figure 6. 1***). It is therefore hypothesised that polysaccharide-specific antibody has a synergistic effect on the bactericidal activity of protein-specific antibody.

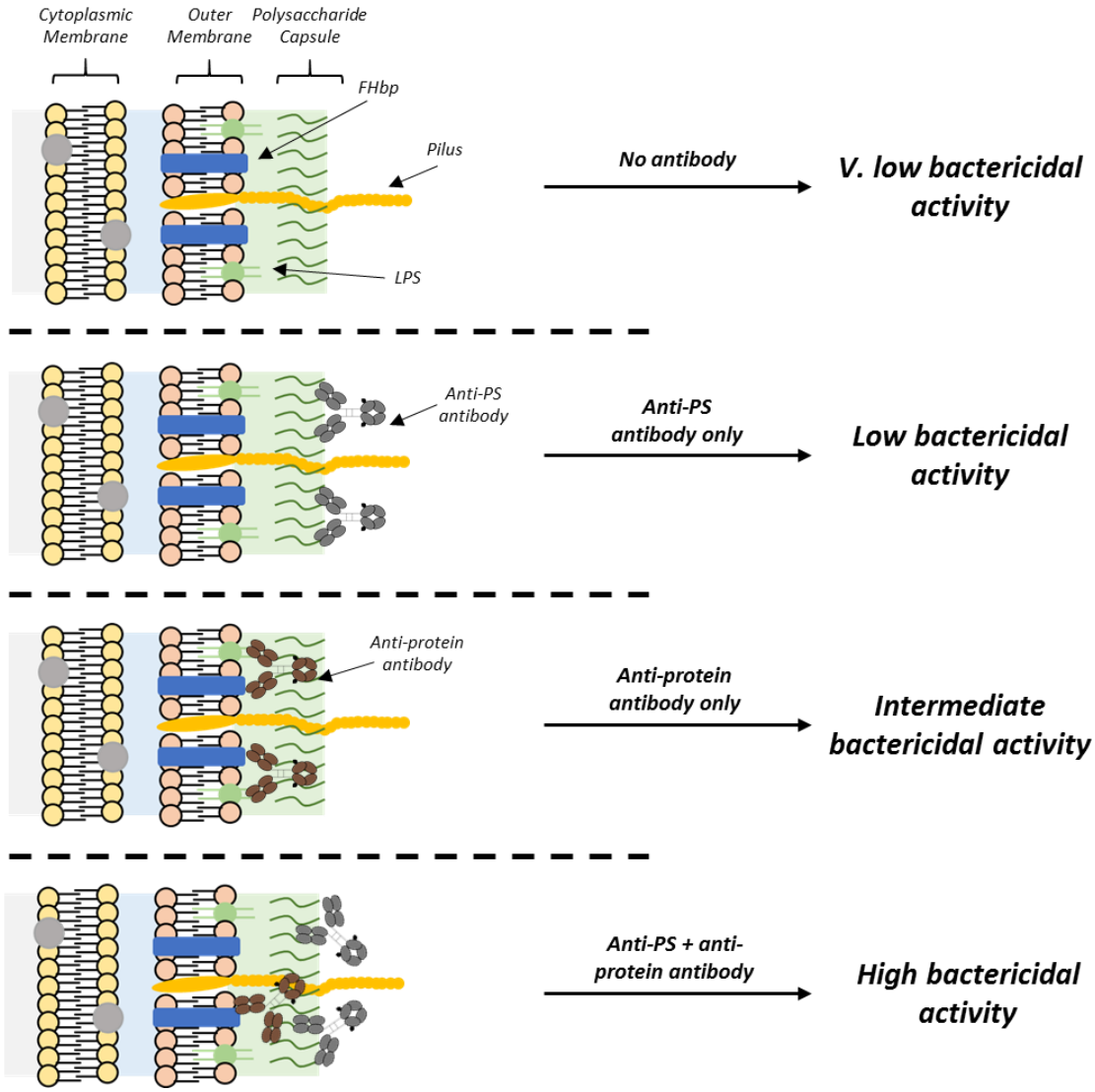


Figure 6. 1 Bactericidal Activity of Purified Polysaccharide-Specific Antibodies
The concentration of meningococcal polysaccharide-specific IgG1 and IgM antibody significantly correlates with the bactericidal of immune serum towards *Neisseria meningitidis*. However, purified polysaccharide-specific antibody is poorly bactericidal. Purified meningococcal protein-specific antibody is significantly more bactericidal than polysaccharide-specific antibody. It is concluded that protein-specific antibody is necessary for polysaccharide-specific antibody to fix complement effectively on the surface of *Neisseria meningitidis*. Polysaccharide-specific antibody has a synergistic effect on the bactericidal activity of protein-specific antibody.

These data suggest that prior exposure to *Neisseria meningitidis* is necessary for successful immunisation with meningococcal polysaccharide conjugate vaccines and may explain why some individuals with seemingly large antibody responses to vaccination achieve poor bactericidal titres (***Chapter 3: Figure 3.9*** and ***Figure 3.13***) (King et al., 1996; Mitchell et al., 1996). Whilst meningococcal polysaccharide conjugate vaccines are effective, these data also suggest that protein-based meningococcal vaccines will produce higher bactericidal titres even in those individuals with no previous exposure to the bacteria. However, due to the variable nature of these antigens it is predicted that meningococcal protein vaccines will have significantly reduced strain coverage compared to meningococcal polysaccharide conjugate vaccines (Vogel et al., 2013). A vaccine consisting of both meningococcal proteins and polysaccharides will most likely produce higher bactericidal titres than the polysaccharide only counterparts.

6.2 Interaction of Human and Rabbit Complement Pathways with *Neisseria Meningitidis*

SBAs using rabbit serum as the source of complement commonly produce much higher titres in those using human serum (Borrow et al., 2001a; Jodar et al., 2000; Maslanka et al., 1997; Zollinger and Mandrell, 1983). Previous studies have shown species-specific interaction of complement regulators with *Neisseria meningitidis*. Specifically, the outer membrane protein FHbp binds human FH but not rabbit FH (Granoff et al., 2009). As such, the alternative pathway of human complement, and not rabbit complement, is actively regulated by the bacteria. The relative contribution of rabbit complement pathways to complement activation on *Neisseria meningitidis* has not previously been investigated, but is hypothesised that the alternative pathway of rabbit complement contributes significantly more to complement activation than the alternative pathway of human complement.

The contribution of the lectin, alternative and classical pathways to complement activation on *Neisseria meningitidis* serogroup W-135 (strain 102/98) in the presence of human serum and BRS was invested using a series of pathway-specific inhibitors (***Chapter 5: Figure 5.15***, ***Figure 5.17*** and ***Figure 5.18***). Blocking the alternative pathway of rabbit complement significantly reduced complement activation. In contrast, blocking the alternative pathway of human complement had little impact on complement activation thus confirming that the alternative pathway of rabbit complement contributes significantly more to complement activation on *Neisseria meningitidis* serogroup W-135 (strain 102/98) than the alternative pathway of human complement.

It has been previously shown that human FH bound to *Neisseria meningitidis* is able to regulate both human and rabbit complement (Granoff et al., 2009). We have shown that the addition of human FH to the source of rabbit complement reduces the SBA titre of vaccinee sera to levels comparable to when human serum is used (***Chapter 5: Figure 5.19***). The addition of human FH to rabbit complement in SBAs will somewhat normalised hSBA and rSBA titres, significantly reduce the number of false positives and ultimately improve the scrutiny of clinical trials assessing the efficacy of new meningococcal vaccines. The same effect may also be achieved with the addition of an alternative pathway inhibitor such as the anti-FB antibody used in this study (Subías et al., 2014). In a similar vein, human FH transgenic animals have resulted in the development of suitable animal models that may be used to assess the potential of new meningococcal vaccines pre-clinically (Granoff et al., 2009; Vu et al., 2012). Whilst the addition of human FH to rabbit serum may improve the specificity of the rSBA, it does not address the issue of the differential ability of human antibody subclasses to activate human and rabbit complement as investigated in this study. One potential solution to this is currently being explored by the Taylor Group at PHE. Endogenous bactericidal activity to *Neisseria meningitidis* is removed from human serum by fully depleting IgG by injecting serum over protein G (Brookes

et al., 2013). This means that large volumes of human serum can be prepared for use in SBAs, removing the requirement for use of rabbit serum.

With the inclusion of FHbp in the recent generation of protein-based meningococcal vaccine antigens, FHbp has been studied extensively (Gorringe and Pajon, 2012; Jiang et al., 2010; McNeil et al., 2013). *Neisseria meningitidis* expresses many other proteins that interact with the complement system of the host. These include PorB2, PorB3 and NspA which also bind FH, NalP which cleaves C3 into an inactive C3b-like fragment and a C3a-like fragment, and PorA which binds the classical and lectin pathway regulator C4BP (Giuntini et al., 2015; Jarva et al., 2005; Lewis et al., 2010; Lewis et al., 2013). It is known that FHbp is specific for human FH and does not bind rabbit FH and that NalP cannot inactivate rabbit C3 (Del Tordello et al., 2014; Granoff et al., 2009). This species-specific complement regulation is thought to account for the far higher bactericidal activity of rabbit serum compared to human serum. The species-specificity of the other FH binding proteins (PorB2, PorB3 and NspA) and the C4BP binding protein (PorA) has not been investigated but would be of great interest in understanding the differences between hSBA and rSBA titres.

Single nucleotide polymorphisms present in genes coding for FH and FHR-3 are associated with increased susceptibility to meningococcal disease (Consortium, 2010). FHR-3 is a truncated homologue of FH lacking both co-factor and decay accelerator activity. FHR-3 competes with FH for FHbp binding and it is thought that the relative serum levels of each either increase or protect against risk of disease (Caesar et al., 2014). Relatively higher levels of FHR-3 binding to FHbp than FH will reduce survival of *Neisseria meningitis* is serum decreasing an individual's susceptibility to disease. Whether FHR-3 competes with FH for binding to PorB2, PorB3 and NspA has not been investigated but may be vital in the understanding of protection against meningococcal disease.

6.3 Conclusions

In this project, I investigated the mechanisms responsible for differential bactericidal activities of human and rabbit complement against *Neisseria meningitidis*. We have described two predominant mechanisms responsible: the differential ability of human antibody subclasses to fix human and rabbit complement and species-specific differences in the interaction of each pathway of complement activation with *Neisseria meningitidis* (***Figure 6. 2***). Species-specific differences in the interaction of each pathway of complement activation with *Neisseria meningitidis* is responsible for the greatly elevated bactericidal titres achieved in SBAs using BRS as the complement source compared to those achieved using human serum. The differential ability of human antibody subclasses to fix human and rabbit complement is responsible for the poor correlation between hSBA and rSBA titres. The data from this project further question the use of rabbit serum as the source of complement in SBAs and provide significant insight into difficulties and challenges associated with interpretation of rSBA data.

This study is of particular importance and relevance due to the recent increase in the incidence of invasive meningococcal disease caused by the hypervirulent serogroup W sequence type 11 complex. In the UK, the number of confirmed cases of meningococcal disease caused by serogroup W has increased from 22 in 2009/2010 to 176 in 2014/2015 despite an overall decrease in meningococcal disease (Ladhani et al., 2015; Ladhani et al., 2016). In 2014/2015, serogroup W accounted for 24.3% of invasive meningococcal infections compared to just 2.6% in 2009/2010. As a countermeasure, two quadrivalent meningococcal conjugate polysaccharide vaccines (Nimenrix™ and Menveo™) were introduced into the 2015 vaccination schedule (Lloyd et al., 2015). It is likely that other regions (such as South America, Europe, South Africa, China and United States), experiencing a similar increase in the incidence of meningococcal serogroup W disease, will also introduce quadrivalent vaccines into their public vaccinations programmes (Ladhani et al., 2015; Mustapha et al., 2016).

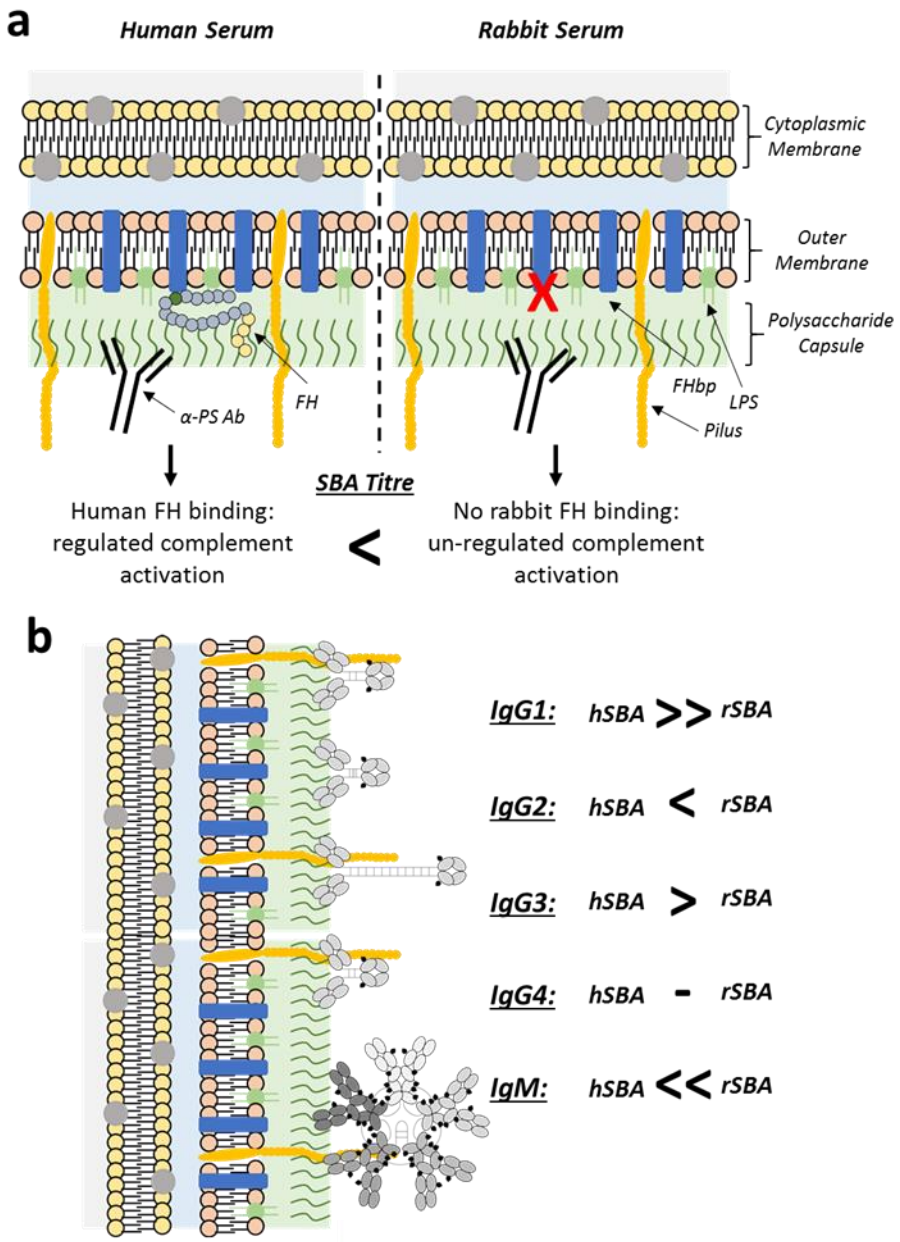


Figure 6. 2 Mechanisms Responsible for Differential Bactericidal Activities of Human and Rabbit Complement against *Neisseria meningitidis*.

Bactericidal titres achieved in SBAs using rabbit serum (rSBA) as the source of complement are significantly higher than and correlate poorly with bactericidal titres achieved in SBAs using human serum (hSBA) as the source of complement. Two mechanisms are proposed for the differential bactericidal activities of human and rabbit complement against N*eisseria meningitidis*. ***a***, The outer membrane protein FHbp of *Neisseria meningitidis* binds human FH but not rabbit FH. As such, the alternative pathway human complement and not rabbit complement is regulated reducing hSBA titres in relation to rSBA titres. ***b***, rSBA and hSBA titres correlate poorly due to the differences between the ability of human antibody subclasses to activate human and rabbit complement. A greater IgG2 and IgM antibody response to meningococcal vaccines produces disproportionately increased rSBA titres compared to hSBA titres whereas a greater IgG1 and IgG3 antibody to meningococcal vaccines produces disproportionately increased hSBA titres compared to rSBA titres.

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